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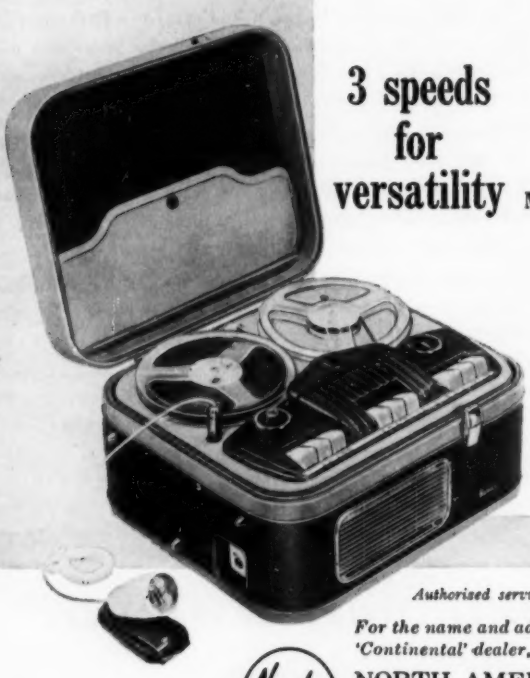

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Fibrous Tissue in Nutritional Cirrhosis

Autoradiographic Studies with Use of Tritiated Thymidine

RICHARD A. MacDONALD, M.D., and G. KENNETH MALLORY, M.D., Boston
With the Technical Assistance of Jane M. Donelan, A.B.

One intriguing and unsolved problem in human and experimental liver disease concerns the origin of fibrous tissue in nutritional cirrhosis. Perhaps the most popular idea in this country at present is one advanced by Hartroft, in which the fibrous tissue that occurs in livers that progress through fatty infiltration to cirrhosis is considered to be derived only from collapsed and condensed reticulum.[1,2] According to this idea, based on studies of rats fed a choline-deficient diet, liver cells fill with fat and form fatty cysts, which rupture, and the supporting reticulum surrounding the cysts becomes condensed. With the formation of new liver cells that in turn become filled with fat and rupture, bands of reticulum are formed that become progressively thickened and elongated. Fibroblastic proliferation and formation of collagenous tissue by fibroblasts have not been considered to be of importance in this concept.[1,2] This has been based on several observations and interpretations. One is the absence of mitotic activity in fibroblasts in the fatty liver.[1] Another is the absence in these livers of fibroblasts with typical stellate processes such as those seen in granulation tissue, and, finally, the interpretation has been made on morphological grounds that the elongated nuclei seen so frequently in early fibrous bands in experimental cirrhosis represent either atrophic nuclei of liver cells that have ruptured and become collapsed, proliferating bile duct epithelium of ductules, endothelial cells, or Kupffer cells.[3] In histological and histochemical studies, it has not been possible to establish conclusively the nature of these nuclei, and their interpretation at present is based chiefly upon morphological characteristics and upon the training and study of different examiners.

Our own interpretation of the fibrous tissue, both in humans with fatty nutritional cirrhosis and in rats fed a choline-deficient diet, has been that many of the elongated nuclei represent fibroblasts and that the absence of stellate processes on these fibroblasts is probably due to their compression by surrounding fat-filled liver cells. Because of the numbers of such fibroblasts and their appearance at the stage when the fatty liver is giving rise to cirrhosis, we consider it likely that fibroblastic proliferation and formation of collagenous tissue by fibroblasts, in addition to condensation of stroma, are important in the pathogenesis of nutritional cirrhosis. In the present study some evidence seems available for

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From the Mallory Institute of Pathology, Boston City Hospital, and the Departments of Pathology of Harvard Medical School, Tufts University School of Medicine, and Boston University School of Medicine.

this interpretation and appears to stand against the interpretation that these nuclei represent atrophic hepatic-cell nuclei, atrophic Kupffer cells, endothelial cells, or bile duct epithelium. Rats maintained on a choline-deficient diet were given injections of tritiated thymidine, a deoxyribonucleic acid (DNA) precursor, at a stage when fibrosis was occurring in the liver. Since DNA is confined to the nucleus [4] and is considered to be formed largely, if not exclusively, by cells undergoing division or preparing for division,[5,6] within minutes of the time of injection [7] this method permits the detection of cells preparing to undergo division to a degree not otherwise possible. Autoradiography was employed as a means of detecting "labeled" nuclei that had taken up the injected thymidine. In the fibrous bands, the elongated nuclei under discussion were observed to show considerable uptake of thymidine and hence to be forming DNA, indicating that they are not atrophic and inactive.

Review

In briefly reviewing the subject of fibrosis in the cirrhotic liver, it should be noted that available information has not been obtained through the application of new techniques. Most statements concerning the subject have been based on histological observations, and these have been subject to varying interpretations. This is true even at the present time.

In experimental cirrhosis due to carbon tetrachloride, repeated administrations of the agent are made at closely spaced intervals so that hepatic necrosis without complete regeneration occurs.[8,9] In this type of cirrhosis, there is both considerable condensation of stroma and fibroblastic proliferation.[10-12] Fibrosis develops in central areas where there is necrosis, probably due to condensation, and in portal regions where there is little or no necrosis, and here it appears to be due to fibroblastic proliferation. In the cirrhosis that follows experimental ligation of the bile ducts, Cameron has pointed out that well-marked cirrhosis follows only if infection occurs and that fibroblastic proliferation is responsible for the resulting fibrosis.[12,13] MacMahon et al., in earlier work, did not feel that infection was responsible for the cirrhosis but emphasized the role of fibroblasts in the development of fibrous tissue.[14,15] In experimental cirrhosis due to fatty infiltration of the liver, such as that produced by a choline-deficient diet, it is of interest that before Hartroft's view became popular various workers described fibroblastic proliferation in these livers and ascribed fibrosis to both fibroblastic proliferation and condensation of existing stroma.[16] Mallory [17,18] used various diets and toxic substances and several species of animals and believed that in cirrhosis both in animals and in humans the fibrous tissue was derived both from condensed stroma, in areas where liver cells developed necrosis or atrophy, and from fibroblastic proliferation, which arose to support nodules of regenerating parenchyma. He believed that fibroblasts gave rise to collagen and elastic fibrils. Connor [19,20] based his observations upon studies of dogs fed alcohol and upon studies of the livers of humans dying with cirrhosis. He described proliferation of fibroblasts and some condensation of stroma. Connor and Chaikoff [21] and Graef et al.,[22] working with dogs, noted cirrhosis following fatty infiltration and without apparently preceding fatty infiltration.[23] Both described cellular fibrous tissue and illustrated what appear to be proliferating fibroblasts.[23] György and Goldblatt, working with rats on a vitamin-deficient diet, ascribed cirrhosis to both condensation of stroma and proliferation of fibrous tissue replacing degenerated and necrotic tissue.[24,25] Wahi produced fatty liver in rats with a deficient diet high in carbohydrate and low in protein and ascribed the developing fibrosis to fibroblastic proliferation.[26]

Materials and Methods

1. Male Sprague-Dawley rats * were housed in individual cages in an air-conditioned room and were fed ad libitum a choline-deficient diet similar to that previously described in detail, with the exception that peanut meal was used in place of arachin.[27] Animals were started on the diet at the age of 59 days. Littermates of the same sex were used as controls. Tritiated thymidine was injected when the animals had been on the diet for periods of four to eight months. A total of 21 rats was used in this study, 13 as test animals and 8 as controls.

2. Single and, in some cases, multiple injections of tritiated thymidine † were given intraperitoneally in a dosage of 1.0μc. per gram of body weight, and animals were killed by decapitation at various intervals after injections, although a standard time of four hours was usually employed. As controls for animals on a choline-deficient diet, littermates of the same age and sex that had been maintained on a standard diet of Purina Lab Chow and water ad libitum were treated in an identical manner.

3. Tissues were fixed in cold 10% neutral buffered formalin and in Zenker's solution. After fixation for 24 hours they were washed in running water for 24 hours, dehydrated, cleared, and imbedded in paraffin. Sections were cut at 4μ-6μ and stained with hematoxylin and phloxine, Feulgen's method for DNA, methyl green-pyronin stain for ribonucleic acid (RNA), phloxine methylene blue, periodic acid-Schiff reagent (PAS), Mallory's aniline blue stain for connective tissue, a modification of Bielschowsky's silver stain for reticulum, Van Gieson's stain for connective tissue, and Sudan black B and oil red O stains for fat. For autoradiography, stripping film technique similar to that described by Doniach and Pelc[28] was employed. Film ‡ was applied over unstained sections and over sections stained with PAS or Feulgen's method. Preparations were exposed in a dry light-tight box at 10 C for 3 to 4 weeks, then developed in Kodak D-19 developer for 7 minutes, fixed in Kodak acid fixer for 10 minutes, washed in cool running tap water for 1 hour, and mounted in polyvinylpyrolidone (PVP).§

* Purchased from the Charles River Breeding Laboratories, Cambridge, Mass.

† Purchased from Schwarz Laboratories, Mount Vernon, N. Y.; specific activity, 360μc. per millimole.

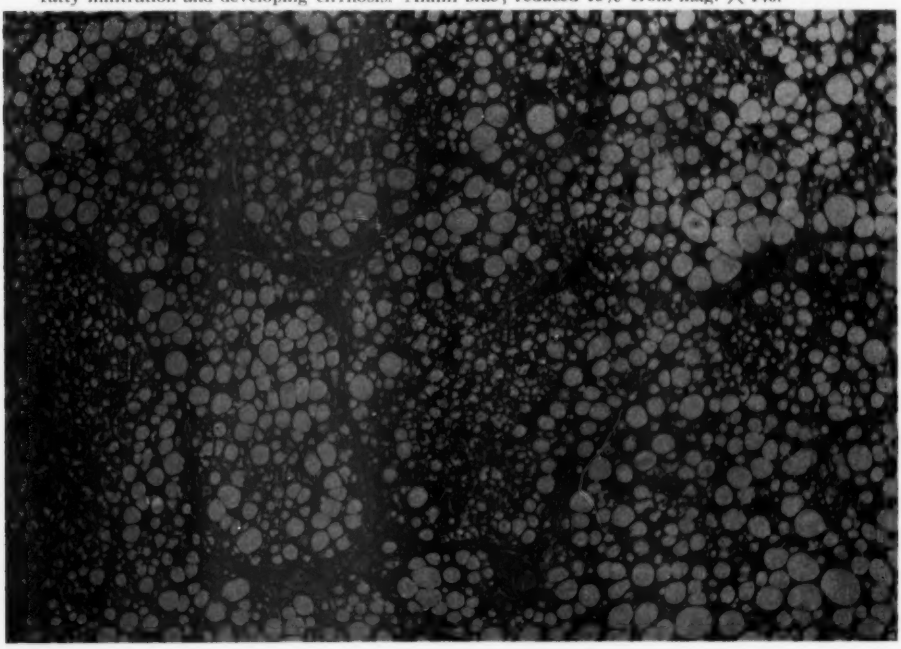
‡ Kodak Limited, London; Film AR 10.

§ Antara Chemical Company, New York.

Results

In animals maintained on the choline-deficient diet pronounced fatty liver devel-

Fig. 1.—Liver of 290 gm. 460-day-old rat fed a choline-deficient diet for 437 days. Note fatty infiltration and developing cirrhosis. Anilin blue; reduced 15% from mag. × 140.



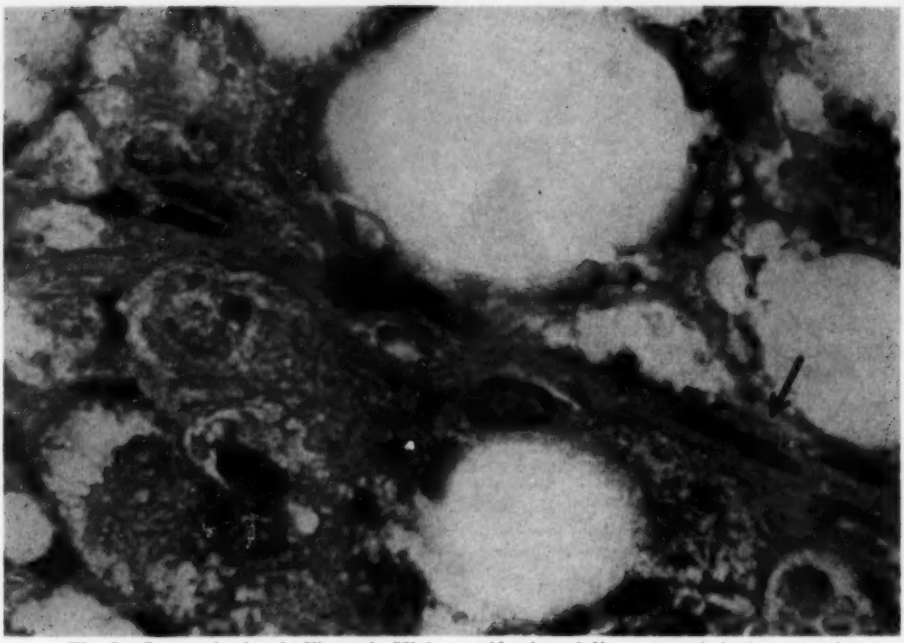
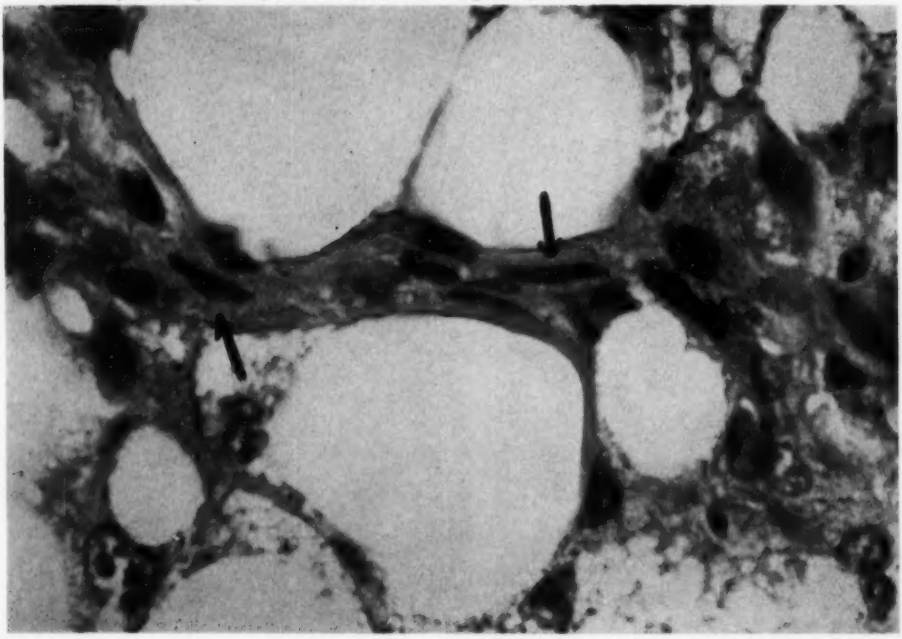


Fig. 2.—Same animal as in Figure 1. High magnification of fibrous strands in liver, showing elongated nuclei of cells considered to be fibroblasts. Hematoxylin and phloxin; reduced 15% from mag. × 1,800.

Fig. 3.—Same as Figure 2, showing additional elongated cells interpreted as fibroblasts. Hematoxylin and phloxin; reduced 15% from mag. × 1,800.



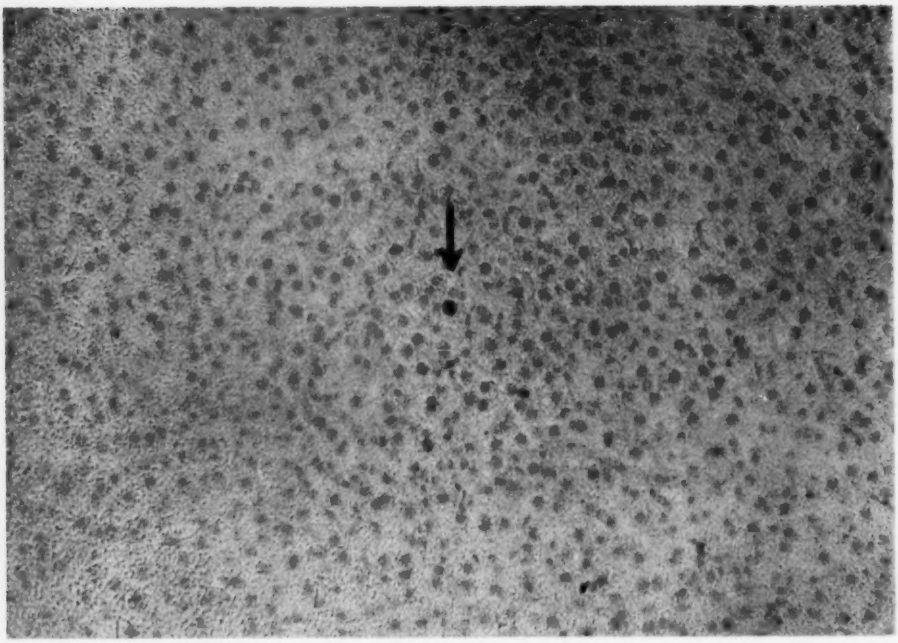
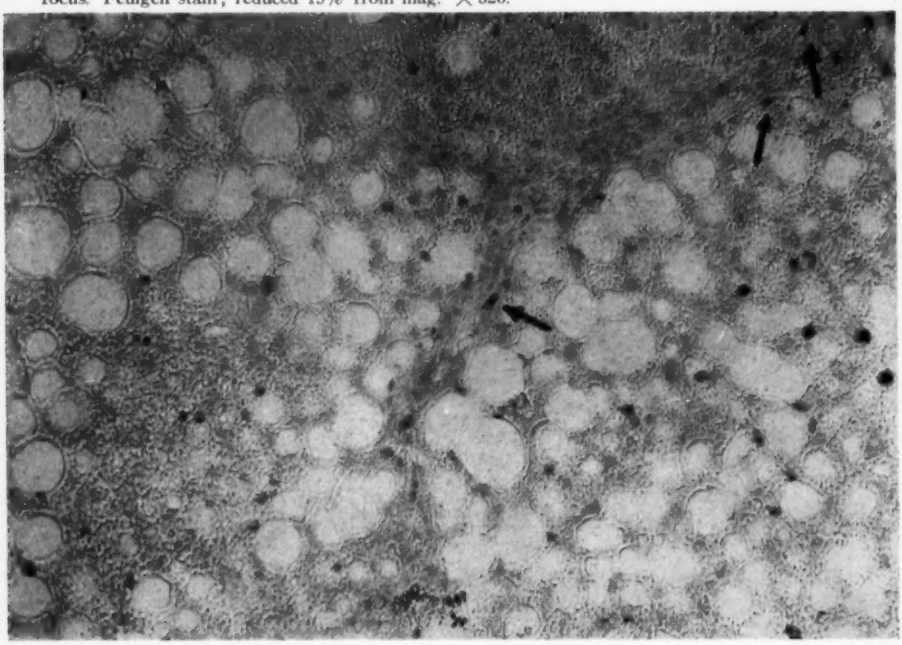


Fig. 4.—Autoradiograph of liver of 140 gm. 48-day-old rat fed a standard diet of Purina Lab Chow, showing normal extent of labeling after single intraperitoneal injection of tritiated thymidine, 1.0μc. per gram. Note single-labeled nucleus in center of field. Feulgen stain; reduced 15% from mag. × 320.

Fig. 5.—Autoradiograph of liver of same rat as shown in Figures 1-3. Note extensive fibrous bands (arrows). Tissue beneath stripping film is of necessity slightly below plane of labeling of hepatic nuclei of many cells filled with fat and labeling of elongated nuclei in focus. Feulgen stain; reduced 15% from mag. × 320.



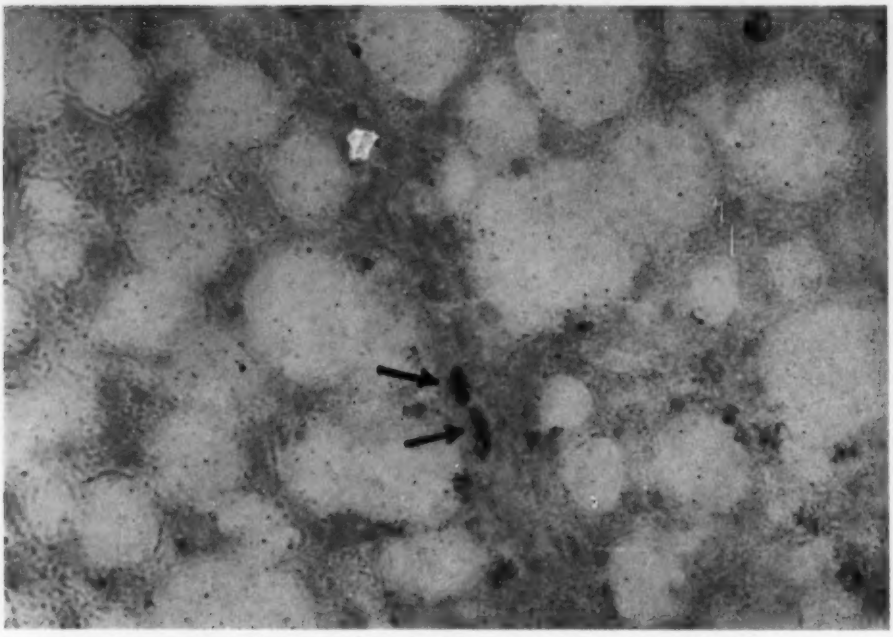


Fig. 6.—Autoradiograph of same liver as in Figure 5, showing labeled cells in fibrous strands. Feulgen stain; reduced 15% from mag. × 760.

oped in approximately three weeks, and after approximately four to six months fibrous strands began to develop in these livers (Fig. 1). This progressed to well-marked cirrhosis in approximately eight to nine months and developed in much the same manner as has been previously reported.[1,2,27] With autoradiographic methods, livers with fat invariably showed a marked increase in labeled hepatic-cell nuclei, at least 5 to 10 times greater than normal (Figs. 4-6). Surprisingly, nuclei contained within the walls of fatty cysts frequently showed labeling, in addition to those of other hepatic cell nuclei. Another striking observation was the marked labeling of elongated nuclei within fibrous strands (Figs. 5 and 6). These nuclei were enclosed within the fibrous strands and were not considered to be either bile duct epithelium or endothelial cells. With aniline blue and Van Gieson stains, the fibrous tissue surrounding these and similar nuclei stained positively for collagen. With reticulum stains, bile duct epithelium in fibrous strands and endothelial cells could usually be identified by their basement membrane, which took a definite silver stain.

Comment

The present studies indicate that livers of rats maintained on a choline-deficient diet undergo a striking degree of proliferative activity when they fill with fat that is not obvious with employment of usual histologic techniques and is only poorly detected by searching for mitoses. This observation is incidentally of interest because of the development of neoplasms in livers of rats maintained on a choline-deficient diet for long periods of time[29] and because of the documented association between cirrhosis and primary hepatic carcinoma.[30] It has long been a matter of discussion whether the stimulus to hepatic carcinoma follows when cirrhosis is well developed or is present concurrently with the development of cirrhosis. From the

present observations, the factors leading to both cirrhosis and carcinoma may be present concurrently, and excessive proliferative activity may lead to neoplasm.

Contrary to what might be expected, hepatic-cell nuclei within the walls of fatty cysts are not dead or inactive cells but are also undergoing constant and considerable proliferative activity. This appears to be inconsistent with the concept that liver cells are taking part in a somewhat passive process in becoming filled with fat until rupture occurs. It is possible that the multiple nuclei contained in walls of fatty cysts arise from the same parent cell by multiple nuclear divisions rather than from fusion of several cells. If this is true, with rupture of a fatty cyst the amount of reticulum forming condensed stroma would probably not be so great as if many individual cells had contributed their reticulum. In addition, rupture of fatty cysts may not be so constant and frequent as previously supposed. Instead, as cells fill with fat they may undergo nuclear division, forming new nuclei and new cytoplasmic structure to accommodate and encompass the increasing amount of fat in the cytoplasm.

The elongated nuclei seen in strands of fibrous tissue, which we interpret as fibroblasts, show striking formation of DNA, which is against the concept that these represent atrophic, compressed, hepatic-cell nuclei. These labeled cells do not appear to be bile duct epithelium, endothelial cells, or Kupffer cells. In additional studies of bile duct epithelium in normal and fatty livers extensive labeling has not been found,[31] although occasional labeling of bile duct epithelium occurs at the stage when cirrhosis is developing in the fatty liver. By comparison with Kupffer cells elsewhere in the same liver, these elongated nuclei interpreted as fibroblasts appeared too large to be nuclei of atrophic Kupffer cells. Similarly, they did not appear to be bile ductules or endothelial cells.

The present studies do not prove the importance of fibroblasts in producing fibrous tissue in experimental nutritional cirrhosis because it is still not possible to identify beyond question the nature of the nuclei in the fibrous strands. However, the demonstration that the nuclei of cells comprising fatty cysts and those enclosed within fibrous tissue are undergoing a striking degree of proliferative activity introduces new information into studies of the cirrhosis resulting from a choline-deficient diet. This may require alterations in the concept that fatty cysts and condensed reticulum alone are responsible for the fibrosis seen in these livers. It appears more likely that both condensation of stroma and fibroblastic proliferation are responsible for the formation of fibrous tissue. The stimulus to the considerable proliferative activity in hepatic cells and to the elaboration of fibroblasts in the present experiments is not known. Further experiments are being carried out to study this point. Another question that arises concerns the origin of the fibroblasts in these livers. This is also not known, and it may be pointed out that biologists and anatomists are not in agreement concerning the origin of fibroblasts in most organs. Evidence has been presented that these cells arise from histiocytes,[32] lymphocytes that are transformed into macrophages,[33] and endothelial cells.[34]

Summary and Conclusions

Rats were maintained on a choline-deficient diet for four to eight months, at which time fibrosis and cirrhosis were developing. At the stage of early fibrosis tritiated thymidine, a deoxyribonucleic acid (DNA) precursor, was injected intraperitoneally and the livers were studied by histologic and autoradiographic methods. Cells preparing for mitotic activity within minutes of the injection were detected in this way. Hepatic cells, including those within the walls of fatty cysts, were observed to be undergoing a striking degree of proliferative activity. Elongated nuclei within fibrous bands, interpreted as fibroblasts, were also undergoing marked proliferation, indicating that these nuclei are not atrophic

and inactive as has previously been supposed.

It is suggested that fibroblastic proliferation as well as condensation of stroma is responsible for the fibrous tissue that occurs in nutritional cirrhosis.

Photographs were made by Leo Goodman.

Mallory Institute of Pathology, Boston City Hospital (18).

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Fibrosis of the Breast

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It is generally accepted that lobular hyperplasia is a mammary dysplasia affecting stroma as well as epithelial elements; one uncommon variant consists of a pure stromal lesion without epithelial hyperplasia or cyst formation. The fundamental picture consists of a dense and amorphous deposition of collagen within the mammary stroma. There have been a few references to this lesion; it was mentioned by Cheatle and Cutler [1] as "fibromatosis," and they suggested a relationship to mazoplasia. Stewart [2] discusses it under the term "chronic indurative mastitis." In a recent text book of pathology [3] the lesion is described as "fibrosis of the breast" and is related to cystic disease and adenosis due to endocrine imbalance. The fullest description of the clinical and pathological aspects is by Haagensen,[4] who calls it "fibrous disease of the breast"; he expresses the belief that it is caused by some form of hormonal dysfunction.

The frequency of this lesion is difficult to assess, but it probably occurs more frequently than generally realized, perhaps because the gross and microscopic changes are very unimpressive. Actually, there are some aspects of the lesion that bear a resemblance to the parenchymal changes of the breast that occur in postmenopausal women. Thus, Maximow,[5] in discussing postmenopausal mammary gland involution, describes the interstitial tissues as undergoing a loss of cellularity associated with transformation of stromal collagen into a homogeneous mass, a process that bears some similarity to the findings in fibrosis of the breast. Cheatle and Cutler also remark on the close histological similarity. The Department of Pathology, Vancouver General Hospital, examines yearly some 500 breast biopsy specimens both grossly and microscopically; approximately half prove to be benign lobular hyperplasia. We have collected 20 examples of fibrosis of the breast from our files. The average age of the patients with this lesion was 33 years, the youngest being 17 and the oldest, 49. The lesion presents as a palpable mass, usually well localized and occasionally slightly tender, most frequently located in the upper outer quadrant of the breast. On palpation, the lump has a moderate firmness and may be mistaken for carcinoma; one lesion caused dimpling of the overlying skin. Usually the lump is unilateral, although occasionally it is found to be bilateral. It is almost never more than 5 cm. in diameter, and usually measures about 3 cm. in greatest dimension. However, in two of the patients simple mastectomy was performed for widespread involvement of the breast parenchyma. Of interest is the fact that it does not often appear to be associated with epithelial hyperplasia or cyst formation; in other words, when present, it usually appears as an isolated lesion. Rarely, however, microcystic duct ectasia may be found.

We have not attempted a detailed clinical investigation of possible associated hormonal imbalance in this group of patients. However, we have the impression that a number do have some history of menstrual irregularity and sterility.

Materials

Multiple sections have been examined, with use of the standard hematoxylin and eosin staining technique, for the 20 cases of fibrosis and 5 representative cases of mazoplasia, 50 normal autopsy

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specimens in different age groups, and 200 specimens of benign cystic disease. Detailed histochemical analyses were performed on five cases from each group and on the five cases of mazoplasia. Hydroxyproline and hexosamine estimations were performed on one representative sample from each group. Acetic acid solubility assays were performed on representative samples from five cases in each group.

Methods

1. *Processing of Tissues.*—All tissues were fixed in 10% formol-saline and processed by the routine laboratory method with use of graded alcohols for dehydration and chloroform for clearing. Specimens were embedded in paraffin wax.

2. *Staining.*—All staining procedures were carried out on adjacent serial sections of each specimen for comparison purposes. With the exception of toluidine blue staining (described below), the methods used were those described by Culling[6]: (*a*) hematoxylin and eosin stain; (*b*) hematoxylin and Van Gieson stain; (*c*) Masson's trichrome stain; (*d*) Mallory's trichrome stain; (*e*) Lillie's allochrome stain; (*f*) Gomori's silver impregnation method for reticulin; (*g*) Gomori's aldehyde fuchsin method; (*h*) Lison's alcian blue stain; (*i*) toluidine blue stain.

The following modification of the toluidine blue staining method was employed in this study. The stain is prepared by dissolving 0.25 gm. of toluidine blue in 100 ml. of Michaelis's barbital (Veronal) acetate-hydrochloric acid buffer at pH 4.5.

Technique: 1. Bring section to water. 2. Stain for 10 seconds. 3. Rinse rapidly in distilled water. 4. Blot, allow to dry, and immerse slide in xylene. 5. If section is not completely clear, repeat Stage 4.

This method was found to be a considerable improvement over previous metachromatic staining methods. Control sections of aorta, cartilage, and inflammatory scar tissue showed clearly defined areas of metachromasia.

Acetic acid-solubility studies of collagen were based on the method described by Banfield.[7]

Hydroxyproline assays were based on a modification of the method of Neuman and Logan.[8] Hexosamine estimations were based on the method described by Blix.[9]

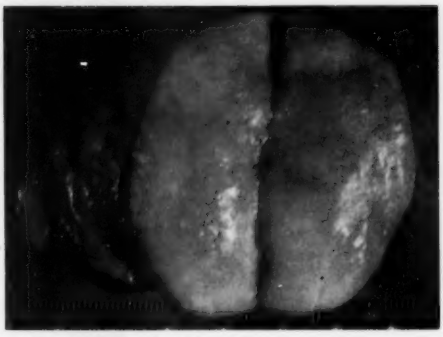

Fig. 1.—Fibrosis of the breast, gross specimen.

Pathological Features

Grossly, the lesion consists of a rubbery uniform mass of yellowish-white tissue that is characteristically nonencapsulated, although well localized within the breast tissue (Fig. 1). When the mass is cut, a rubbery firmness is encountered but not grittiness or cyst formation.

Microscopically, the mass consists of an amorphous acellular and avascular deposition of collagen within the stroma. Large fields of this fibrous tissue may be seen to be virtually devoid of any epithelial com-

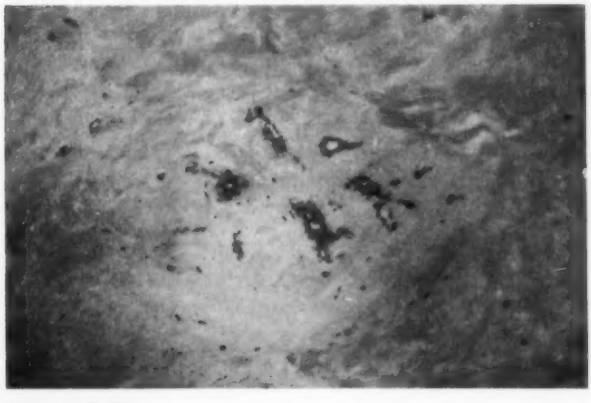

Fig. 2.—Collagenous obliteration of gland field; × 110.

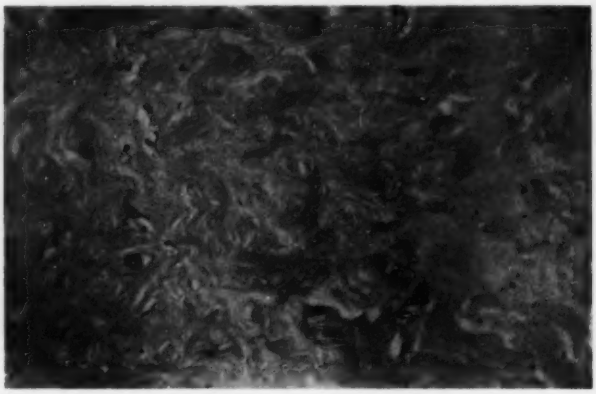

Fig. 3.—Representative field of stromal fibrosis; × 110.

ponent; indeed, in some areas small remnants of epithelial lobules may be seen to be undergoing complete obliteration by the collagenous overgrowth (Figs. 2 and 3). Mazoplasia, on the other hand, exhibits quite a different microscopic picture in which prominent gland fields are accompanied by an extravagant loose and cellular, rather myxomatous, intralobular stroma; epithelial activity is present, and some intraluminal secretion may be observed (Fig. 4).

The findings, by use of the variety of histochemical stains, may be summarized as follows: The trichrome stains revealed apparently normal collagen in all tissue examined from each group. The only significant finding with the reticulin stain was the presence of fine reticulin fibrils lying in the intralobular stroma in the cases of mazoplasia only. These fibrils were present in those areas in which ground substance was demonstrable. The toluidine blue stain revealed, in cases of mazoplasia only, very prominent intralobular metachromatic ground substance with some apparent "seepage" into the interlobular stroma. After treatment with hyaluronidase (150 turbidity units per milliliter of 0.3% sodium chloride for 18 hours) these areas revealed no evidence of metachromatic staining. Sections of normal control breasts showed only rare and widely dispersed lobules exhibiting minimal intralobular metachromasia; sections of typical foci of benign cystic disease, similarly, did not contain significant amounts of detectable metachromasia. In none of these tissues was mast-cell or fibroblast activity a prominent feature, nor was metachromasia of blood

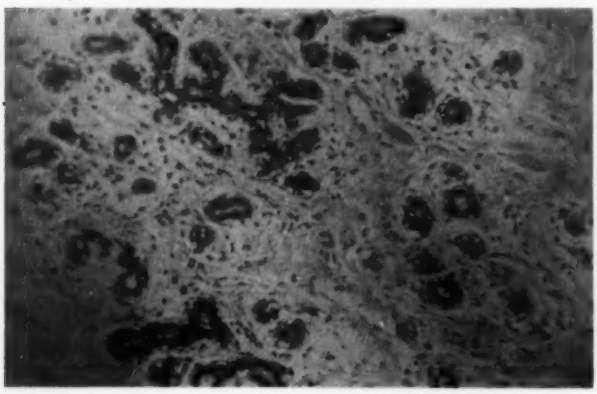

Fig. 4.—Representative gland field of mazoplasia; × 110.

vessel walls apparent.[10] Lillie's allochrome stain revealed the presence of periodic acid-Schiff (PAS)-positive material within the intralobular stroma in cases of mazoplasia only. Gomori's aldehyde fuchsin stain revealed no significant difference in the elastic tissue pattern between the various groups examined. However, it was noted that those areas staining metachromatically with toluidine blue also exhibited a typical pale purple coloration with use of the aldehyde fuchsin method. Control sections of aorta and inflammatory scar tissue, before and after treatment with hyaluronidase, confirmed this apparent specificity of staining reaction. In fact, this technique appeared to have considerable value in the rapid location of metachromatic ground substance. These findings were also confirmed by the use of Lison's alcian blue staining method for acid mucopolysaccharides.

These histochemical findings are not in complete agreement with those described by Ihnen and Perez-Tamayo in their study on changes in the breast stroma.[11] They found metachromasia more constantly present in the interlobular collagen rather than within the loose matrix of intralobular stroma. This may be due to the use of alcohol in their toluidine blue staining technique, since Pearse[12] has shown that the use of alcohol after toluidine blue staining produces variability of metachromasia, with reversion to the orthochromatic form of toluidine blue.

In three cases (Table) chemical analyses of the mammary parenchyma were performed. The results suggest that the collagen content of a collagenous mass is qualitatively unaltered compared with normal breast collagen, although, of course, the total content of the mass is increased. On the other hand, the hexosamine level in mazoplasia appears increased, thus reflecting the increase in periductal ground substance.

Chemical Analyses of Mammary Parenchyma

	Hydroxyproline *	Collagen *	Hexosamine *
Normal control	9.94	74.2	7.3
Fibrosis	9.47	70.7	6.1
Mazoplasia	8.0	59.5	10.0

* Reported as milligram per gram of dry fat-free tissue.

Finally, acetic acid-solubility tests were performed on representative samples of collagenous mammary stroma in normal controls of different ages, in fibrosis and in benign cystic disease of the breast. Unfortunately, acetic acid-solubility studies were not performed in cases of mazoplasia. The results of these studies indicated virtually complete lack of soluble collagen in all tissues examined, for never more than a 1+ precipitation of reconstituted collagen was obtainable. Harkness et al.[13] suggest that the acid-soluble collagen fraction may represent the most recently deposited collagen. Our results would indicate, therefore, that recent fibroblastic activity is absent.

Comment

The histochemical and biochemical analyses of this lesion indicate that the process consists of a deposition of excessive quantities of collagen within the breast stroma. The mode of origin of this collagenous tissue is uncertain, although two of these cases present suggestive evidence that preceding focal mazoplasia may play a predominating role in their development. These two cases, clinically and grossly, appeared to represent excellent examples of mammary fibrosis. However, microscopically, they both had features of mazoplasia[1,17] characterized by prominent pericanalicular and perilobular stroma containing fibroblastic activity and deposition of mucopolysaccharide ground substance. It seems likely that this periepithelial activity has acted as a precursor to the development of the dense and amorphous collagenous mass which has obliterated virtually all of the gland fields within its vicinity. Similar changes are to be seen in an older well-developed fibroadenoma or gynecomastia. In this respect, the histochemical studies of Fisher and Creed[14] are of interest, for they demonstrated the presence of increased ground substance in

the loose myxomatous periductal stroma in gynecomastia and fibroadenoma. The close relationship between fibroblastic activity and mucopolysaccharide ground substance production has been well demonstrated by Taylor and Saunders.[15] There is evidence that the periductal stroma participates in the cyclical menstrual changes in the breast,[16,17] and it seeems likely that this stromal stimulation is exaggerated to some degree in the so-called "mazoplasia," described by Cheatle and Cutler, and in "mastodynia," described by Geschickter.[17] In normal breasts the cyclical premenstrual changes within the intralobular stroma consist of nothing more than a slight transudation of edematous ground substance. This transudate is rapidly resorbed, however, before collagenization of the ground substance can occur.

The sequence of events in the development of this lesion may well start with cyclical (physiological ?) mazoplasia, followed by a focus of mammary parenchyma going into an irreversible noncyclical phase in which continuous periglandular fibroblastic activity eventually leads to deposition of excessive stromal collagen. In this respect, the description of mazoplasia in Anderson's textbook of pathology[18] is of interest, the gross and microscopic features corresponding to what we believe represents the later phases of mazoplasia undergoing fibrosis. Whether this process can truly be termed "pathological" may be debatable; probably minor degrees of it are within the variations of normal physiological activity. However, the fact that the patient and the surgeon can feel a definite palpable mass in the breast and that excision biopsy of this mass may be performed leads us to believe that wider recognition of the process is necessary.

Experimental work is accumulating on the relationships of hormone effects with ground substance and collagen production. This work supports the concept that deposition of collagen within the mammary stroma is probably under direct or indirect hormonal control. Of considerable interest is the well-demonstrated effect of estrogens on connective tissues; for example, Burack et al.[19] have shown that prolonged estrogen administration in the rat causes transformation of reticulum into collagen and continued deposition of collagenous tissue in the genital tract. Furthermore, they note that the effects of estrogens appeared to hasten the aging processes in the genital tract. They found[20] that, as age advanced, there was an increase in the amount of condensation of the collagenous tissue in the genital tract of the normal rat. We may speculate that mammary fibrosis may be somewhat analogous and represent a focus of hormonal imbalance, such as relative or periodic hyperestrogenism, giving a histological picture similar to that seen in aging. Arcadi[21] has related this estrogenic effect to decrease in ground substance solubility, probably due to increased polymerization. Certainly there is a decrease in collagen solubility with age, as measured by hydrolysis with collagenase[22] and acetic acid solubility.[7] Of further interest are the experimental findings[23] which suggest that the estrogenic effects on the breast are dependent upon an intact pituitary gland. Also, it has been shown that estrogens stimulate peritubular fibroblastic activity and collagen deposition in the human testis,[24] the end-result being sclerotic acellular tubules. On the other hand, androgens cause an increase in metachromatic ground substance (accompanied by an increase in hexosamine content) in the cock comb.[25] Finally, Geschickter,[17] in describing lobular premenopausal involution of the breast, compares the stromal condensation of collagen with the experimental production of similar changes in rats on a high-estrogen intake and progressively diminishing doses of luteal hormones.

Summary and Conclusions

A review of 20 cases of fibrosis of the breast is presented. It is believed to represent a form of benign lobular hyperplasia

and to have a similar origin in hormonal imbalance, probably hyperestrogenism.

Chemical and histochemical analyses of these lesions reveal them to be composed of apparently normal collagen, although of a rather sclerotic nature, similar to that seen in prolonged hyperestrogenism or aging.

The morphogenesis of this lesion is discussed, and the possibility is entertained that focal mazoplasia precedes the fibrosis. The sequence of events is believed to be initiated by excessive and prolonged intralobular ground substance deposition, with subsequent transformation into collagenous tissue.

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Addendum

Since completion of this manuscript Ozzello and Speer [26] have published a histochemical study of the breast; they, too, remark on the failure of intralobular stroma to respond to cyclic alterations in mammary dysplasia.

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Chronic Encephalopathy Following Minor Head Injury

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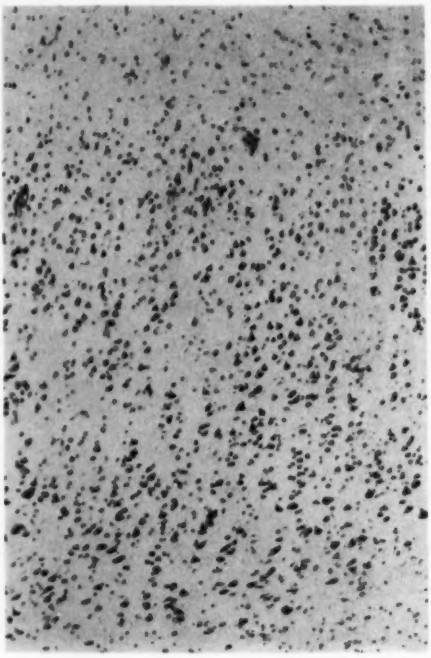
When the head is endangered in sporting activities, serious and diverse complications may arise from seemingly trivial trauma. Among the rare injuries, a form of chronic encephalopathy associated with boxing and known as dementia pugilistica has been documented anatomically in recent years.[1-3] The purpose of this report is to describe another type of long-standing diffuse cerebral degeneration which resulted from an apparently minor head injury incurred in a wrestling match.

Report of Case

A 17-year-old previously healthy Negro boy was reported to have struck his head during a supervised wrestling match at school. It caused no concern at the time. The next day, although he seemed to be well, he declined to participate in sports. The following morning he was found in bed unconscious. He lay motionless and mute for about two months, and then he began to respond slightly. X-rays of the skull and the spinal fluid at this time and later were normal. Five and one-half months after the injury he was admitted to Colorado General Hospital. He appeared to be alert but moved little. He was able to swallow, to recognize his name, and to parrot phrases, but he could not speak sentences. He responded to pain, made clucking noises, and often clicked his teeth together. He had assumed the decerebrate position of extensor rigidity and maintained it during the entire illness. He developed contractures of the hands, arms, and feet and finally decubital ulcers. The external ocular muscles, the pupils, and the fundi were normal. The deep-tendon reflexes were hyperactive and symmetrical. The psychiatrist stated that the patient appeared to be fairly alert but seemed unable to answer questions or to cooperate and exhibited marked echolalia and some negativism; he was uncertain whether the symptoms were functional or organic in nature. A pneumoencephalogram in the seventh month revealed symmetrical dilatation of all ventricles and a large amount of air in the subarachnoid space, which was felt to indicate rather severe cortical atrophy, especially in the temporal region. Periodic fevers were attributed to infections of the urinary tract. He was discharged home for custodial care, where he died 13½ months after the onset. Significant autopsy findings were limited to the central nervous system.

Brain.—The embalmed brain weighed 1,035 gm. A firm brown membrane no more than 1 mm. thick adhered to the inner surface of the dura mater over much of the convexity of the hemispheres. There was no evidence of accumulation of fluid blood or of cyst formation in the subdural space. The leptomeninges were unaltered except for mild brown tinging. The convolutional pattern was

Fig. 1.—Temporal cortex showing mild focal diminution in the number of nerve cells. Nissl stain; × 90.



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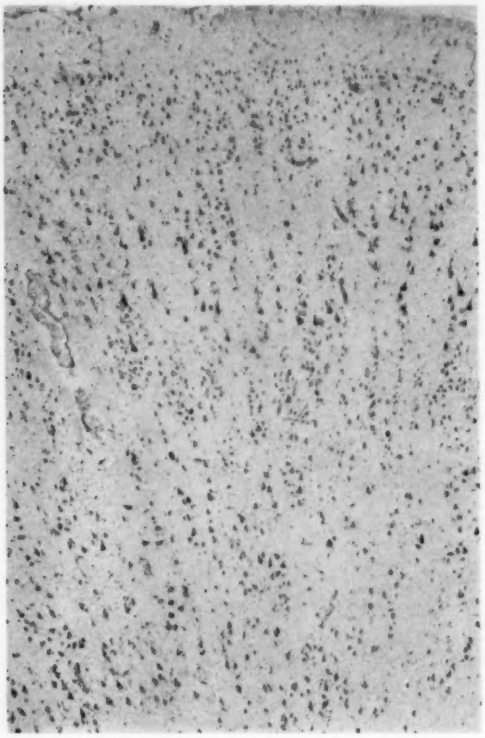

Fig. 2.—Parietal cortex showing loss of nerve cells in all layers. Nissl stain; × 60.

normal, but the gyri were somewhat atrophic, especially over the frontal lobes. The central white matter was shrunken but normal in color and consistency, and it was free from focal lesions. The corpus callosum was very thin and soft. There was advanced bilateral atrophy of the caudate nuclei and rather less severe atrophy of the putamens. Small brown softened patches were present in the globus pallidus on both sides. The ventricles were moderately dilated.

Microscopically, the dura was slightly fibrotic, with numerous aggregations of hemosiderin cells and stripe-shaped bands of fibroblasts and lymphocytes on the inner surface. The cerebral cortex in most places exhibited diffuse thinning, with general preservation of the stratification but with minute poorly defined areas of neuronal outfall. Occasional nerve cells showed chronic nonspecific degeneration. A mild to moderate diffuse plasmatic gliosis was noted. Changes in the white matter were more impressive. Extensive incomplete demyelination with an infiltration of transparent macrophages involved the deep white matter in large poorly delimited patches but spared the arcuate fibers. This was accompanied by proliferation of astrocytes and oligodendroglial cells, the presence of some typical gitter cells, sometimes in perivascular location, and slight fibrillary gliosis. With hematoxylin and eosin, the advanced state of the demyelination was not apparent but the fibers were separated by minute, clear locules. This spongy reticulation was due to the presence of the phagocytes filled with lipoid demonstrable with the scarlet R and Sudan IV staining methods. Compactly massed refractile unstained crystals were observed throughout the white matter in frozen sections. The axis cylinders exhibited fragmentation, swelling, and reduced staining qualities in the most severely damaged areas, but in general they were much better preserved than the myelin sheaths.

Fig. 3.—Reticulated convolutional white matter with many phagocytic cells and a dilated perivascular space. Hematoxylin and eosin; × 175.

Lesions were accentuated in the corpus callosum. The number of neurons was reduced in the caudate nuclei. Less severe atrophy was noted in the putamens. The globus pallidus showed advanced focal softening, with hemosiderin pigmentation and beginning cyst formation. The thalamus, midbrain, and cerebellum were not remarkable. Mild demyelination and fibrillary gliosis secondarily affected the pyramids and cervical cord.

Comment

This was an unusual sequel of trauma to the head. Up to the time of death it was uncertain whether the disease resulted from concussion or encephalitis. The lesions were undoubtedly traumatic, in light of the pigmentation of the meninges with iron. The hemorrhages, contusions, and lacerations which commonly attend violence to the skull are well understood. Delayed traumatic apoplexy, central traumatic softenings, dementia pugilistica (punch-drunkenness), and cortical atrophy are much rarer complications. The atrophy in this case, which was demonstrated by pneumoencephalogram and at autopsy, must have been due mainly to shrinkage of the white matter, although there was unmistakable atrophy of the cortex. Focal lesions were limited to the globus pallidus. The subdural hemorrhage probably played a minor role, possibly interfering with the absorptive activity of the Pacchionian granulations and aggravating the accumulation of cerebrospinal fluid. The amount of the hemorrhage was such that serious compression of the cortex probably had not occurred. Furthermore, central demyelination was the one outstanding lesion.

This syndrome, so far as we know, is comparable only to that described by Strich.[4] She reported five patients with

Fig. 4.—Focally demyelinated cortex at the top, relatively well-preserved arcuate fibers, and demyelinated deeper white matter. Spielmeyer stain; × 10.

closed-head injuries without fracture of the skull. Unconsciousness immediately followed the trauma. None had intracranial hemorrhage or evidence of cerebral edema. They survived for 5 to 15 months with spastic quadriplegia. They were wakeful but unresponsive and demented. Three had attacks of decerebration. Grossly, the brains exhibited only dilatation of the ventricles and occasional small incidental softenings and hemorrhages. Diffuse alteration of the white matter resembling Wallerian degeneration, with loss of nerve fibers and the presence of gitter cells, were the principal changes. The severity of the process was appreciated only with the use of the Marchi method, and the lipoid was not stainable with Sudan IV. Secondary degeneration of the thalamus and asymmetrical deterioration of the long descending tracts were observed. Strich believed that anoxia, edema, vascular disturbances, and fat embolism were not significant in the pathogenesis. She favored the assumption of direct mechanical damage, with stretching and tearing of the nerve fibers and vessels accompanying rotation which produced shearing strains and distortion of the brain.

The lesions in our patient differed somewhat from those of Strich's patients. The cerebral cortex was atrophic; the demyelination was severer and less discrete; the axis cylinders were better preserved, and prominent cystic softenings involved the basal nuclei. The symptoms also were delayed. It seemed unlikely that all of these lesions were attributable entirely to the immediate effects of direct force.

We believe that edema developed in the white matter and was later followed by a phagocytic reaction and by gliosis. Jacob[5] described diffuse destruction of the white matter subsequent to edema from various causes. Such damage is widespread, but it often spares the arcuate fibers and periventricular white matter. It is independent of the irrigation areas of large vessels, and it is characterized by severe involvement of the myelin sheaths and better preservation of the axis cylinders. There is much plasmatic and less fibrillary gliosis, and there is little mesenchymal activity. These features tally with those seen in our case. The significance of edema following head injury also was emphasized by Greenfield,[6] who stated that edema rather than the blow itself may cause areas of demyelination in the centrum semiovale; this lesion may be found months and years after a severe cerebral contusion.

In addition to liberation of fluids from the brain substance and vessels, it is conceivable that transient stasis could have played a part. The softenings in the central gray masses of the brain were identical with those previously described in acute rapidly fatal cases of closed-head injury, and they can be understood only as sequels of circulatory disturbances, due to either mechanical compression or functional im-

pairment of larger arterial branches. Pampus and Müller,[7] for example, reported the pertinent case of a young boxer who died 20 days after a knockout and whose brain showed numerous softenings in the extracortical gray matter. Evans and Scheinker [8] also thought that such vascular disturbances were the cause of the alterations of the white matter which they observed in acute traumatic cerebral edema. The lesions in our case were probably preceded by the less severe changes which they detailed; the reticulated appearance and paling of the white matter, the presence of perivascular and pericellular spaces, and minor glial changes.

The explanation of the cortical atrophy meets with more difficulty; it certainly was not secondary to the damage of the white matter. The operation of a thixotropic mechanism was a likely possibility. Hallervorden,[9] and Hallervorden and Quadbeck [10] applied the doctrines of colloidal chemistry to cerebral concussion. They considered that the cerebral protoplasm behaves as a system of labile sols and gels and that trauma could produce an immediate alteration such as liquefaction of the gels (thixotropy), which, if not reversible, might result in serious parenchymal change and atrophy. We believe this theory had merit in accounting for the diffuse degeneration of gray matter in a boy who died 10 months after a single severe closed-head injury.[11] We [3] also agreed with Brandenburg and Hallervorden [1] and with Grahmann and Ule [2] that thixotropy best explained the cortical atrophy of boxers. Lindenberg and Freytag [12] considered thixotropy as a factor in the pathogenesis of cerebral contusions; direct force, by means of this mechanism, caused the necrosis of neurons.

It would seem, therefore, that besides direct concussive damage to the nervous parenchyma a constellation of factors was operative in the development of the complex degenerative process which had resulted from a single blow to the head.

Summary

A chronic encephalopathy with dementia which followed a single minor head injury incurred in a wrestling match is described. It was characterized by severe demyelination throughout the white matter of the cerebral hemispheres. An incidental subdural hemorrhage and small softenings of the basal ganglia were present, but contusive lesions were absent. The demyelination was thought to have developed predominantly on the basis of post-traumatic edema. Thixotropy was considered to be of importance in the explanation of the extensive, but less impressive, cortical atrophy. This case demonstrates the necessity of thorough examination of the white matter in cases of trauma; severe lesions may be detected histologically and may be of far greater significance than grossly more prominent meningeal hemorrhage or small contusions.

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Differentiation of Neoplastic Lesions Characterized by Large Vacuolated Intraepidermal (Pagetoid) Cells

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Large pleomorphic intraepidermal epithelial cells with optically clear cytoplasm constitute an outstanding morphologic feature of such cutaneous lesions as Bowen's disease, Paget's disease (both mammary and extramammary forms), malignant melanoma, and junction nevus. They have also been observed in the anal skin of some instances of rectal carcinoma.[1] Such appellations as Bowen's or Paget's cells, or less committal references as Bowenoid or Pagetoid cells, have been utilized for their designation depending upon the disorder with which they have been found. Although the occasional presence of intercellular bridges in such cells or the occurrence of dysplastic changes in adjacent epithelium may be found only in Bowen's disease, other morphologic features allowing for the differential diagnosis of these intraepidermal lesions are lacking. Even the diagnostic significance of intracellular melanin may be questioned since similar pigment has been observed in some well-documented examples of mammary Paget's disease.[2] Particular diagnostic difficulty may be expected to occur when such cells are encountered in the epidermis of the perianal skin. In addition to the occurrence of Bowen's disease, extramammary Paget's disease, and malignant melanoma at this site, similar epidermal alteration may be noted with some rectal carcinomas [1] and malignant neoplasms considered to arise from the cloacogenic zone of the anorectal junction.[3] Since the differentiation of these lesions is of prognostic and therapeutic importance, methods which provide for their more exact diagnosis would be highly desirable.

We have previously called attention to certain tinctorial features of the large, intraepidermal epithelial cells with optically clear cytoplasm, observed in an example of extramammary Paget's disease, which allowed for their differentiation from the similar-appearing cells of Bowen's disease. These findings also provided information relative to their pathogenesis.[4] More recently we have encountered similar-appearing intraepidermal cells in the perianal skin of a 50-year-old white man in whom a careful pathologic study failed to reveal any alteration of the underlying apocrine glands or anorectal ducts. The tinctorial features of these cells were unlike those observed previously in Bowen's and extramammary Paget's disease or in examples of malignant melanoma with intraepidermal spread. The extension of the process into the anus, including the anorectal junction, suggested the possibility that the origin of these cells was from the mucous-secreting elements described at the latter site.[5] A somewhat similar case has been recently recorded by Rabson and associates.[3]

The purpose of this report is to present the results obtained with a few relatively simple histochemical procedures which allow for the differentiation of the various neoplastic lesions in which these large intraepidermal epithelial cells are observed.

Material and Methods

Sections were prepared in the usual manner from formalin-fixed blocks of examples of malignant melanoma with intraepidermal spread (8), junction nevi (4), Bowen's disease (5), extramammary Paget's disease of perianal skin (1), Paget's

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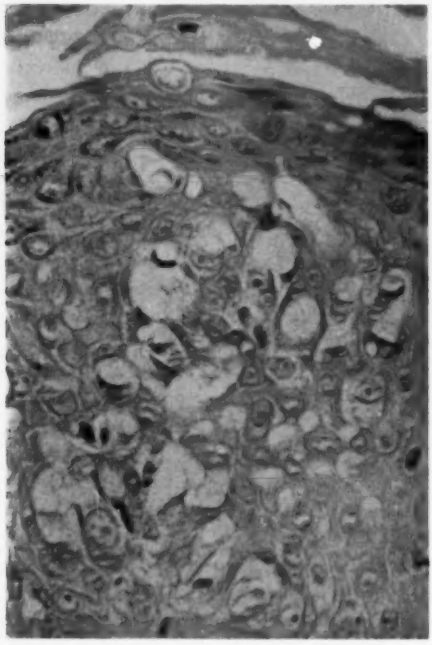

Fig. 1.—Large pleomorphic epithelial cells with optically clear cytoplasm in epidermis of perianal skin from patient with cloacogenic carcinoma. Hematoxylin and eosin; × 370.

disease of the breast (3), senile keratoses (3), nummular eczema (3), and intramucosal cloacogenic carcinoma (1) obtained from a 50-year-old white man who had been treated by various physicians for pruritis ani for three years without appreciable success. Biopsy of involved perianal skin revealed frequent large vacuolated intraepidermal epithelial cells (Fig. 1). Intercellular bridges were not recognized, and adjacent epithelial cells appeared unaltered. Resection of the involved perianal skin and anal mucosa including the dentate line was performed, and a skin graft was applied. Examination of the operative specimen revealed cells in the mucosal epithelium of the anal canal, including the dentate line, which were identical to those observed in the biopsy of the perianal skin. Numerous sections failed to disclose any neoplastic involvement of apocrine glands or perianal ducts.

In addition to the pathologic specimens, sections of normal perianal and axillary skin with apocrine glands, rectal mucosa, and the anorectal junction were similarly prepared. All sections were stained simultaneously by the following methods:

1. Hematoxylin and eosin.
2. Thionin, 1:10,000, pH 4 for one-half hour with and without antecedent treatment with ribonuclease (Nutritional Biochemical Corp.), pH 8.6 phosphate buffer for one hour at 37 C.
3. Periodic acid-Schiff technique with and without antecedent diastase digestion (Fisher) pH 8.6 phosphate buffer, one hour at 37 C.
4. Mowry modification of the Alcian blue technique.[6]
5. Rinehart, Abul-Haj technique.[7]
6. Danielli reaction for tyrosine, tryptophan, and histidine.[7]

Results

The results of the various tinctorial reactions are listed in the Table. Periodic acid-Schiff-positive material was identified in the cytoplasm of many of the large intraepidermal epithelial cells of all lesions studied except the examples of melanoma or junction nevi. These latter frequently contained rust-colored cytoplasmic material in cells with melanin when stained by this technique (Fig. 2). However, a true reaction was not noted. Not all cells appeared positive in Bowen's disease, although little difficulty was encountered in identifying intracytoplasmic Schiff-positive material in

Tinctorial Reactions of Cytoplasm of Large Intraepidermal Epithelial (Pagetoid) Cells *

	PAS	Diastase + PAS	Ribonuclease + Thionin	Alcian Blue	Rinehart, Abul-Haj	Danielli
Mammary Paget's disease	+	+	—	+/—	+/—	+
Extramammary Paget's disease	+	+	—	+/—	+/—	+
Bowen's disease	+	—	—	—	—	—
Cloacogenic carcinoma	+	+	M	+	+	+
Malignant melanoma	—	—	—	—	—	—
Junction nevus	—	—	—	—	—	—
Senile keratosis	—	—	—	—	—	—
Nummular eczema	—	—	—	—	—	—

* PAS indicates periodic acid Schiff; M, metachromatic.

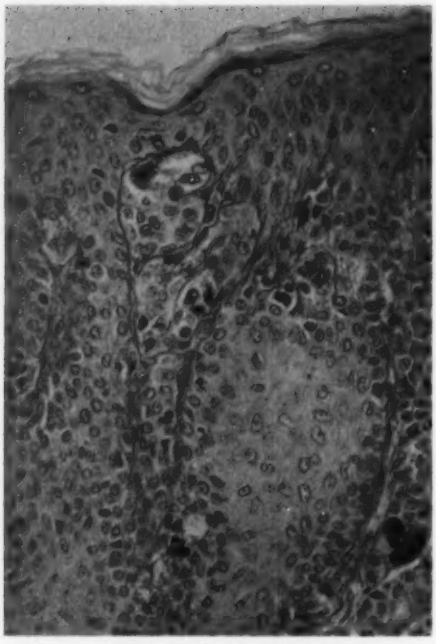

Fig. 2.—Nests of epithelial cells in epidermis of junction nevus. Their cytoplasms are optically clear. Periodic acid-Schiff stain; × 260.

sections of this disorder. The positive periodic acid-Schiff reactions observed were inhibited by diastase digestion only in the examples of Bowen's disease, senile keratosis, and nummular eczema (Fig. 3). On the other hand, only cytoplasms of the intraepidermal cells observed in the example of cloacogenic carcinoma and the goblet cells of normal rectal mucosa and anorectal junction were metachromatic with thionin Fig. 4). The metachromasia observed was resistant to ribonuclease treatment. The latter procedure facilitated the recognition of this reaction by removing the normal cytoplasmic basophilia of surrounding epithelial cells. The cytoplasm of varying numbers of the cells of both types of Paget's disease occasionally exhibited diastase-resistant periodic acid-Schiff droplets. In some instances, these latter were also colored by the Alcian blue and Rinehart methods (Fig. 5). Normal apocrine glands, the apocrine carcinoma, mammary ducts, and associated carcinomas contained fairly abundant diastase-resistant periodic acid-Schiff droplets. Only rare droplets were colored with the Alcian blue and Rinehart methods. These latter reactions appeared most intense in the luminal coagulum and so-called brush border of these structures. In many instances, particularly in sections prepared from the perianal skin, these structures were not colored by these methods. Distinct metachromasia or glycogen could not be demonstrated in the many sections of apocrine glands examined, although the luminal border and coagulum of small mammary ducts were metachromatic.

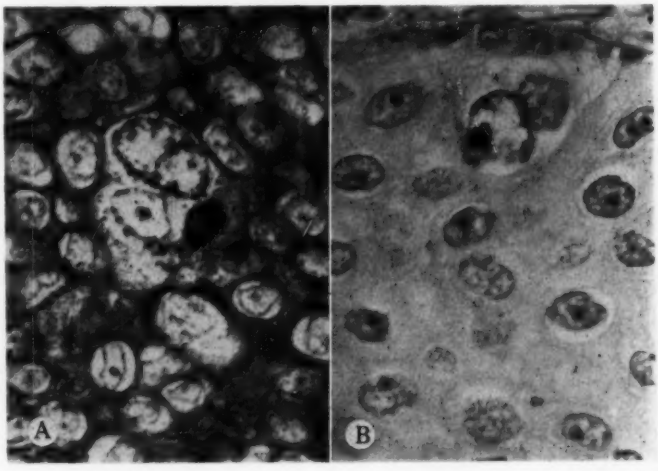

Fig. 3.—*A*, presence of periodic acid-Schiff-positive material in cytoplasm of Bowen's cell and surrounding epidermal cells; × 825. *B*, periodic acid-Schiff-positive material is no longer present after diastase digestion; reduced approximately 30% from mag. × 825.

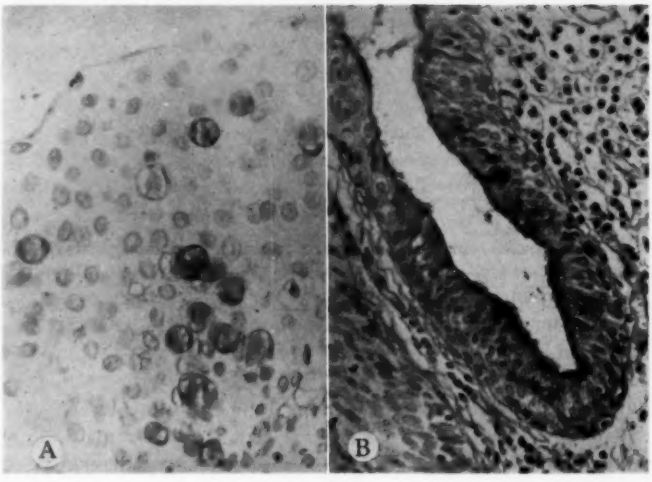

Fig. 4.—*A*, metachromatic material (appearing gray) in cytoplasm of intraepidermal cells in perianal skin in example of cloacogenic carcinoma. Ribonuclease-thionin stain; ×250. *B*, Rinehart-positive material in luminal portions of epithelial cells of cloacogenic zone of control; reduced approximately 30% from mag. ×290.

Glandular structures in the breast, which were morphologically indistinguishable from apocrine glands of the skin, revealed tinctorial reactions identical to the latter.

Comment

The results of this study indicate the value of the application of a few relatively simple histochemical procedures for the identification of the various intraepidermal epithelial cells encountered in Bowen's and Paget's disease, malignant melanoma, and junction nevi, and intraepithelial carcinoma arising in the cloacogenic zone of the anus. The periodic acid-Schiff reaction, performed with and without antecedent diastase digestion, and the demonstration of metachromasia, with thionin or related dyes, appear sufficient to allow for this differentiation. The identification of glycogen within the cytoplasm of some Bowen's cells, as well as similar-appearing cells in senile keratosis and some of the vacuolated cells in nummular eczema, is coincident with the well-recognized occurrence of such material in normal epidermal epithelium. The absence of such material in some Bowen's cells may well reflect cytologic dedifferentiation.

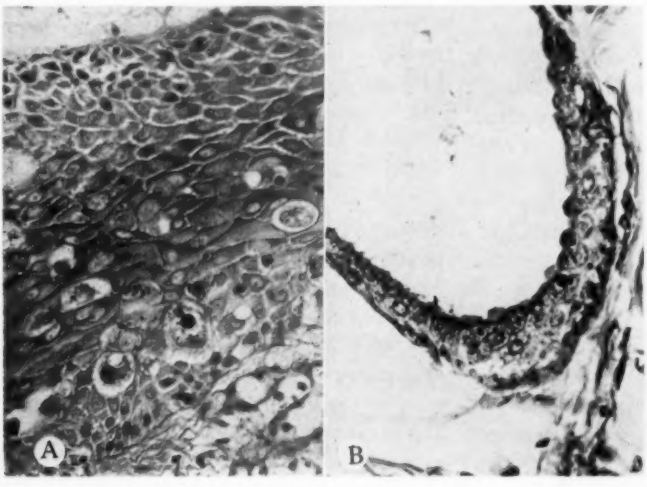

Fig. 5.—*A*, diastase-resistant periodic acid-Schiff-positive cytoplasmic droplets in cells from example of extramammary Paget's disease of perianal skin; ×375. *B*, similar-appearing droplets in epithelium of apocrine gland of control skin; reduced 25% from mag. ×490.

On the other hand, the periodic acid-Schiff-positive material encountered in the cytoplasm of morphologically similar cells in extramammary and mammary forms of Paget's disease is characterized as glycoprotein or neutral mucopolysaccharide, as evidenced by its resistance to diastase treatment and positive protein reaction. In addition, some acid mucopolysaccharide may be present, as indicated by the droplets which are colored by the Alcian blue and Rinehart methods. It is to be noted however, that the specificity of these techniques for acid mucopolysaccharides has been questioned.[9] It appears significant, from a diagnostic as well as a histochemical standpoint, that the material within these cells, as well as that in normal or dilated apocrine glands and the example of apocrine carcinoma, is not metachromatic. Although the failure to demonstrate metachromasia in the epithelial cells or coagulum of normal or dilated apocrine glands is unlike the results of Montagna,[10] a similar experience has been recorded by Bunting and associates,[11,12] who concluded that apocrine glands of the skin as well as the breast were devoid of acid mucopolysaccharide. It also appears significant that Cawley[8] noted the Paget's cells to be orthochromatic in an example of extramammary Paget's disease of the axilla. It is possible that some Paget's cells in the mammary form of Paget's disease may contain metachromatic substance, since small mammary ducts occasionally possess such material, although in the examples studied none was evident. Further, there would obviously be no confusion in the diagnosis of the intraepidermal cells in this lesion with those of cloacogenic carcinoma which present similar tinctorial reactions. It is pathogenetically significant that tinctorially identical material was observed in normal apocrine epithelium as well as the neoplastic cells of the underlying apocrine carcinoma. These findings indicate, as emphasized previously,[4] that such cells represent either a direct extension or intraepidermal metastases from the associated apocrine carcinoma rather than two independent neoplastic processes. A similar interpretation has been offered by Cawley[8] in an example of extramammary Paget's disease of the axilla. The tinctorial reactions observed in the cells of the lesion designated as intraepithelial cloacogenic carcinoma indicate the presence of acid mucopolysaccharide and some glycoprotein and/or neutral mucopolysaccharide, which is similar to that observed in goblet cells of normal rectal mucosa and the cloacogenic zone of the anorectal junction, differing from apocrine glands by being markedly metachromatic. The occurrence of such a lesion at the cloacogenic zone may offer an explanation for those examples of so-called Paget's disease of the anus in which no apocrine gland carcinoma can be identified. Since the term Paget's disease refers to that neoplastic process characterized by the presence of atypical epithelial intraepidermal cells of the type described and their association with a malignant neoplasm of apocrine glands or related structures, we have designated this lesion as intraepithelial cloacogenic carcinoma rather than extramammary Paget's disease. Recently, Rabson and associates[3] have described a somewhat similar case in which neoplastic transformation of apocrine glands was observed in addition to the occurrence of "Paget's cells" within the anal mucosa. They interpreted these findings to indicate multicentric neoplastic transformation of both of these structures. The application of the methods outlined would appear of value in substantiating this contention.

Summary

The utilization of several histochemical procedures has provided information of value for the differential diagnosis of the large pleomorphic intraepidermal epithelial cells with optically clear cytoplasm observed in Bowen's and extramammary Paget's disease, malignant melanoma, and intraepithelial carcinoma arising in the cloacogenic zone of the anorectal junction. The cyto-

plasm of extramammary Paget's cells, their associated apocrine carcinoma, and normal apocrine glands contains glycoprotein and/or neutral mucopolysaccharide, as evidenced by a positive diastase-resistant periodic acid-Schiff reaction as well as a positive protein reaction. In addition, some acid mucopolysaccharide may be present, but it is orthochromatic. Examination of three examples of mammary Paget's disease revealed identical tinctorial reactions, although some terminal mammary ducts reveal intraluminal metachromatic material. Some Bowen's cells, on the other hand, contain intracytoplasmic glycogen, as evidenced by the diastase-labile nature of its periodic acid-Schiff material. Unlike either of the preceding morphologically similar cells observed in an example of intraepithelial cloacogenic carcinoma, normal rectal mucosa and mucous cells of the cloacogenic zone contain metachromatic acid mucopolysaccharide in addition to some glycoprotein or neutral mucopolysaccharide. The pathogenetic implications of these findings are discussed.

Attention is directed to an example of intraepithelical cloacogenic carcinoma with Pagetoid cells.

Recognition by Dr. Raymond Goldblum of Pittsburgh of the unusual clinical nature of the cloacogenic lesion prompted this histological study, and Dr. J. B. Hazard, Pathologist, Cleveland Clinic, permitted us to review the sections prepared from the operative specimen in this case.

Laboratory Service, Veterans Administration Hospital (40).

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Reticuloendothelial Response to Carbon Tetrachloride

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This study was planned to observe the alterations, if any, of the hemopoietic system following administration of a single subcutaneous injection of carbon tetrachloride in rats of the Long-Evans strain. The study correlates the hepatic histological changes during the stages of necrosis, inflammation, regeneration, and hepatic restoration with the imprints of cellular morphology of the lymph nodes, the thymus, the liver, the spleen, the bone marrow, and smears of the peripheral blood. All the animals were autopsied serially at 18, 24, 48, 72, 120, and 168 hours after the subcutaneous injection of a single dose of carbon tetrachloride. The dose utilized was 0.4 ml. of carbon tetrachloride per kilogram of body weight of the experimental animal.

Although many brilliant studies employing many techniques, including spectrophotometry, cytology, chromatography, and enzymology, have been used in ascertaining the degree of liver injury resulting from acute carbon tetrachloride poisoning, nowhere in the literature have we found a systematic report of the impact on the cellular morphology of the hemopoietic system. No reference reveals the use of imprint technique in any study of carbon tetrachloride poisoning. In itself, this would be of interest and value. Correlated with simultaneous observations of the liver pathology it is additionally significant, since until now the literature has stressed only the renal and the hepatic lesion of this classic injury and overlooked the important hemopoietic system. Five tissues—lymph node, thymus, blood, spleen, and marrow—are thus additional witnesses to the dynamic alterations induced in liver and kidney by the injection of carbon tetrachloride.

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The Research Laboratory, Veterans' Administration Hospital and the University of Oregon Medical School.

Methods and Materials

Animals.—Mature rats of the Long-Evans strain were used in this experiment. The control group consisted of 20 animals, and the experimental group, of 16 animals.

Diet.—From the time of weaning until autopsy, animals were on the Rockland Rat Diet (complete), which consists of cane molasses, soy bean meal, fish meal, condensed buttermilk, gluten meal, wheat germ oil, oats, wheat bran, wheat flour middlings, yellow corn, hulled barley, hulled oats, wheat, milk powder, alfalfa leaf meal, A & D Feeding Oil, steamed bone meal, salt, irradiated yeast, linseed oil meal, corn oil meal, and calcium carbonate, fortified with minerals and vitamins.

Environment.—The experimental animals were housed in an air-conditioned room in which the temperature was regulated to 73 to 75 F and the relative humidity at 45% to 50%.

Injection.—One injection of 0.4 ml. of carbon tetrachloride per kilogram of body weight was administered subcutaneously.

Autopsy.—Animals were autopsied serially at 18, 24, 48, 72, 120, and 168 (1 week) hours.

All autopsy procedures were performed with rats under ether anesthesia, and a detailed inspection was made of all viscera. Particular attention was focused upon the liver. A blood smear was obtained from the tail of the animal. All organs were then removed and weighed on a Roller-Smith torsion balance except the liver, which was weighed on an analytical balance. Imprints were studied of the lymph node, thymus, spleen, liver, and marrow. Smears of peripheral blood were made simultaneously at autopsy.

Imprint Technique.—Imprints of femur marrow were made from three areas of the femur across a series of four slides to obtain maximum variation present in any one animal. Imprints of the spleen were obtained from both the lower and the upper poles across a series of four slides. Specimens of thymus, liver, and lymph node were made from a cross section of each organ. The imprinting technique consists of lightly touching the slide with the tissue, held in a pair of forceps, in a straight up-and-down movement, across a

series of slides in all cases. In this manner, one layer of cells adheres to the slide, without distortion, and a series of imprints from different areas of each organ examined are visualized on one slide.

All slides were stained with Osgood's modification of the Wright's technique.

Wright's Stain: National Aniline Wright's Stain C. P., 0.5 gm., is dissolved in 100 ml. of absolute methyl alcohol (acetone-free). It is important to keep the stock bottle of alcohol tightly closed, as contamination by air and water vapors will spoil the stain.

Let stand at room temperature or in a warm dry place for 24 to 48 hours. Stir several times. The stain is then filtered and is ready for use. Keep in a tightly stoppered bottle.

Buffer for Wright's Stain: Weigh out 5.12 gm. of Na_2HPO_4 (dibasic) and 13.26 gm. of KH_2PO_4 (monobasic) and put in a 2.000 ml. volumetric flask. Dissolve in distilled water, and dilute to mark. The pH must be in the range of 6.4 to 6.6.

Wright's Stain Technique: Flood slide with Wright's stain and let stand for three minutes. Add buffer until a metallic sheen appears. Mix thoroughly by tilting rack gently. Set timer. Blood slides are stained 15 minutes. Imprints are stained 30 to 45 minutes.

Method of Examination.—All slides were first reviewed under low power for gross morphology and variations of the imprints on each slide. A more detailed examination and description of the cytology was made under oil-immersion objective. One thousand cells were enumerated on each spleen and marrow. A differential count of 100 cells was performed on each blood specimen. Reticulocyte counts were performed with use of 0.1% brilliant cresyl blue in 0.85% saline. Tail blood and brilliant cresyl blue were mixed in equal quantities and allowed to incubate at room temperature for 3 to 10 minutes. Smears were made, and reticulocytes were enumerated by use of a Miller disk. Observations were made on specimens of liver, lymph node, and thymus without enumeration. It is unfortunate that, because of technical difficulties, the absolute total cell counts cannot be obtained. Hence, only relative and not absolute values were obtained.

Results

Lymph Node.—The normal lymph node of the mature rat consists of a homogenous pattern of mature lymphocytes interspersed with an occasional lymph precursor and a very rare cell of reticuloendothelial origin (Fig. 1). Mast cells, plasmacytes, and eosinophils are not observed in our series of normal rats.

Twenty-four hours after one injection of carbon tetrachloride, a marked proliferation of plasmacytes occurs in the lymph node, with numerous mast cells throughout. Only a rare cell of reticuloendothelial origin is observed (Fig. 2).

However, 48 hours after one injection, numerous cells of reticuloendothelial origin are scattered throughout the entire pattern of the node, both singly and in masses.

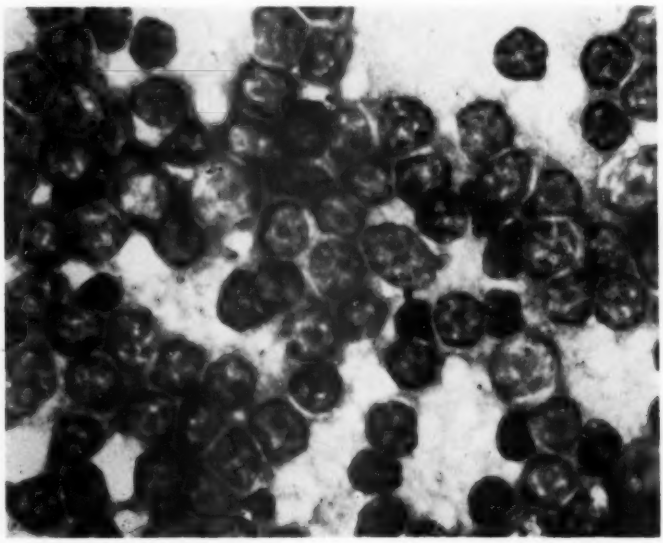

Fig. 1.—Lymph node of the normal rat. Homogenous field of mature lymphocytes; reduced approximately 10% from mag. ×842.

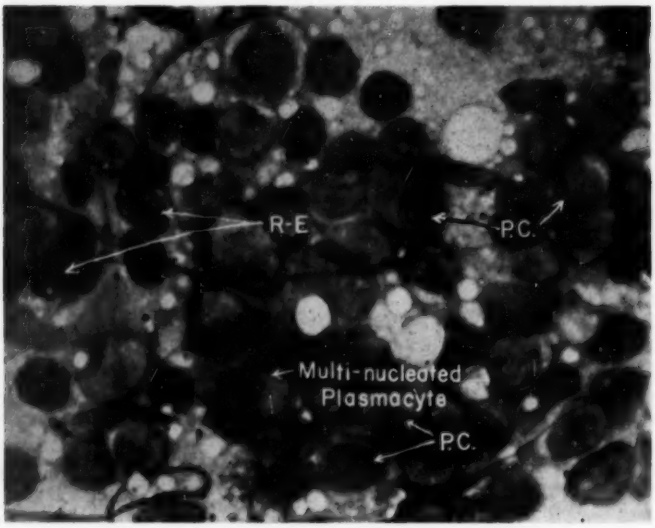

Fig. 2.—Lymph node of the rat 24 hours after one injection of carbon tetrachloride. Marked proliferation of plasmacytes; one is noted with three nuclei; reduced approximately 15% from mag. ×842.

These cells are uninuclear, 15μ to 20μ in size, with one to two prominent blue nucleoli and a vesicular chromatin pattern. An occasional lipophage is noted. Markedly increased proliferation of plasmacytes and mast cells continues from the 24-hour period (Fig. 3).

Lymph precursors are abundant at 72 hours, with only an occasional eosinophil, plasmacyte, and a rare mast cell present. At this stage, reticuloendothelial cells are within normal limits, with only a rare cell of this series observed (Fig. 4).

Again, at 120 and 168 hours, we note increased proliferation of a moderate degree of plasmacytes and mast cells, with occasional eosinophils interspersed throughout the specimen. At these hours, reticuloendothelial cells are within normal limits, both morphologically and numerically (Fig. 5).

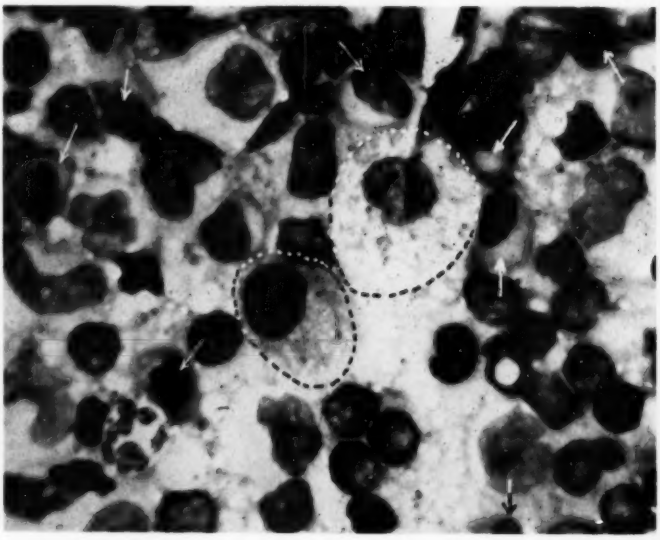

Fig. 3.—Lymph node of the rat 48 hours after one injection of carbon tetrachloride. Delineated cells are of reticuloendothelial origin with large vacuolated cytoplasm. Arrows indicate plasmacytes; reduced 15% from mag. ×842.

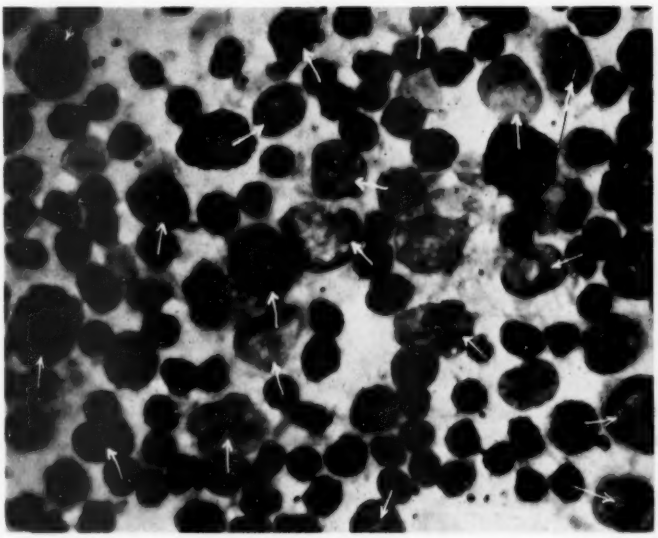

Fig. 4.—Lymph node of the rat 72 hours after one injection of carbon tetrachloride. Marked proliferation of reticuloendothelium. Two mast cells are observed to the left and right of center; reduced 15% from mag. × 842.

Thymus.—The normal mature rat thymus consists of a diffuse even pattern of mature lymphocytes with a very occasional precursor. Cells of reticuloendothelial origin and plasmacytes are rarely encountered.

The normal distribution persists after one injection of carbon tetrachloride at 18 and 24 hours.

However, at 48 hours, an increased proliferation of plasmacytes is observed, which is more pronounced at 72 hours.

At 120 and 168 hours, the plasmacytosis has decreased to near normal levels, with only an occasional plasmacyte remaining. Proliferation of other series was not noted in the thymus at any time in this study.

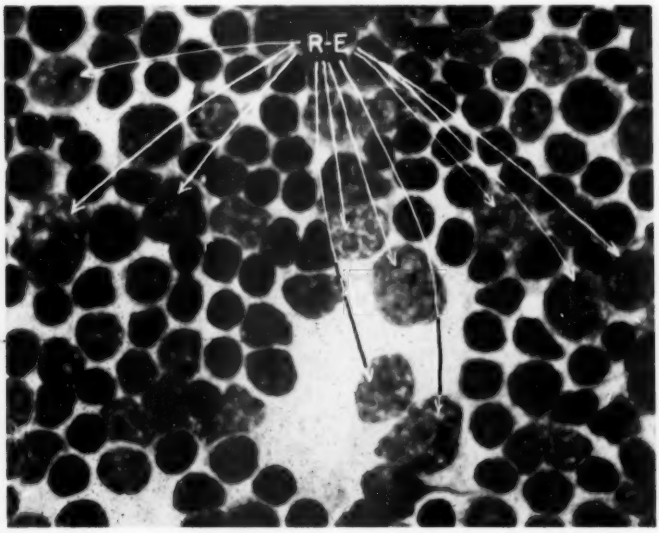

Fig. 5.—Lymph node of the rat 168 hours (1 week) after one injection of carbon tetrachloride. Continued proliferation of reticuloendothelial cells; reduced 15% from mag. × 842.

TABLE 1.—*Distribution of Hemic Population in Acute CCl_4 Toxicity of Mature Long-Evan: Rats*

Time, Hr.	Rats, No.	Total No. of Neut.	Eos.	Mono.	L.	D. C.	P. C.	Rub.	Baso.	Retic., %
18	2	26.5	2.0	7.0	65.0	5.5	0.0	0.0	0.0	—
24	2	30.0	2.5	0.5	60.0	7.0	0.0	0.0	0.0	2.0
48	3	21.0	3.0	5.0	59.0	10.0	0.0	0.0	2.0	1.6
72	4	15.5	4.0	5.0	68.5	6.0	0.0	0.0	0.5	2.1
120	2	4.0	5.5	1.0	84.5	5.0	0.0	0.0	0.0	1.9
168	2	16.0	1.0	1.0	76.5	5.5	0.0	0.0	0.0	1.2
Normal	20	18.6	2.0	1.2	69.6	8.2	0.1	0.4	0.0	1.4

Blood.—Initially, at 18 hours after one injection of carbon tetrachloride, a mild neutrophilia and a more marked monocytosis is observed. The neutrophilia persists at 24 hours, with a return to normal levels of monocytes. Monocytes are again increased at 48 and 72 hours, returning to normal at 120 hours. A straight-line decrease of neutrophils is observed from 48 through 120 hours, at which time they reach a markedly low level; they return to normal levels at 168 hours. During this same period, eosinophils increase, reaching a peak at 120 hours and returning to normal values at 168 hours. A peak of lymphocytes is observed at 120 hours as a relative increase related to the marked neutropenia. Other series remained within normal 95% confidence limits throughout the experiment (Table 1).

Liver.—Liver imprints of the rat under normal conditions reveal a mottled pattern of Kupffer cells, singly and in aggregates. Myelopoiesis, erythropoiesis, lymphopoiesis, or megakaryocytes are not observed.

Liver imprints did not deviate from the normal cytological characteristics in this study.

Spleen.—Cytology of the normal rat spleen, in general, reveals a large preponderance of mature lymphocytes, with an occasional neutrophil and metarubricyte. A rare eosinophil, basophil, reticuloendothelial cell, plasmacyte, and megakaryocyte is encountered in a few animals. Inter-

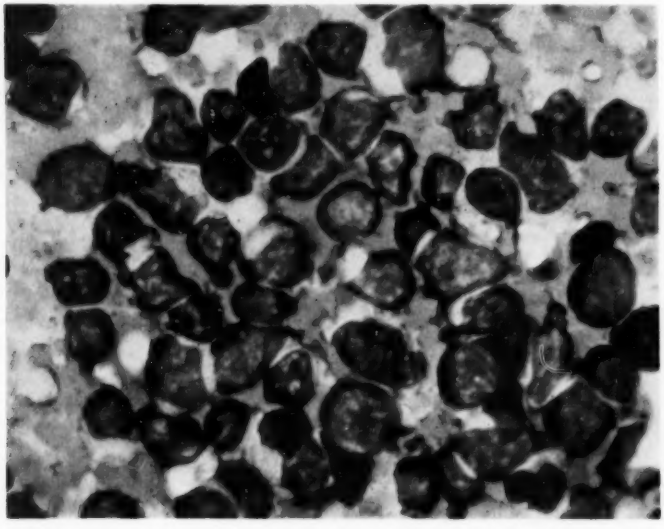

Fig. 6.—Spleen of the normal rat. A homogenous field of mature lymphocytes with an occasional lymphocytic precursor is noted; reduced 15% from mag. ×842.

TABLE 2.—*Distribution of Splenic Population in Acute CCl_4 Toxicity of Mature Long-Evans Rats*

Time, Hr.	Rats, No.	Total No. of Neut.	Eos.	Baso.	R-E.	P. C.	Rub.	L.	Megak.
18	2	3.2	0.4	0.1	2.0	0.5	0.2	93.4	Absent
24	1 *	3.0	1.4	0.0	16.0	1.0	0.0	78.6	Absent
48	2 †	1.4	0.9	0.0	8.0	7.0	0.6	82.1	Absent
72	2 †	0.8	0.4	0.0	13.5	2.3	0.0	83.0	Rare
Normal	20	1.0	0.4	0.02	0.6	0.4	1.9	95.7	Rare

* 2 rats actually observed.
† 4 rats actually observed; 2 rats observed at 120 and 168 hr. } Splenogram not enumerated due to marked number of masses of R-E. present.

spersed throughout are lymph precursors, which appear in minimal numbers (Fig. 6).

A more detailed account of the findings of the normal rat spleen will be published in another communication. Normal findings for a group of 20 control animals are included in the splenic population chart as mean values in relation to those found in our series after a single injection of carbon tetrachloride (Table 2).

Cells of reticuloendothelial origin are approximately 12μ to 15μ in size and possess a deeply basophilic cytoplasm without nucleoli. Reticuloendothelial cells in the spleen are elevated initially, at the first observation of 18 hours, and are markedly increased at 24 hours (Fig. 7).

The level drops somewhat at 48 hours but still remains within the range of a marked increase (Fig. 8).

At 72 hours, this series is again at a very high level, and it remains so throughout this experiment (Chart 1).

As early as 24 hours, an occasional syncytial mass * of these cells is encountered. At 48 hours, syncytial masses were observed in marked numbers in two of four

* Syncytial mass, as referred to in this paper, means a large number of individual cell nuclei present in cytoplasm without any visible individual cell boundaries. This definition does not lend itself to the hypothesis that these are merely clumps of cells, since clumps of cells have readily visible cell boundaries.

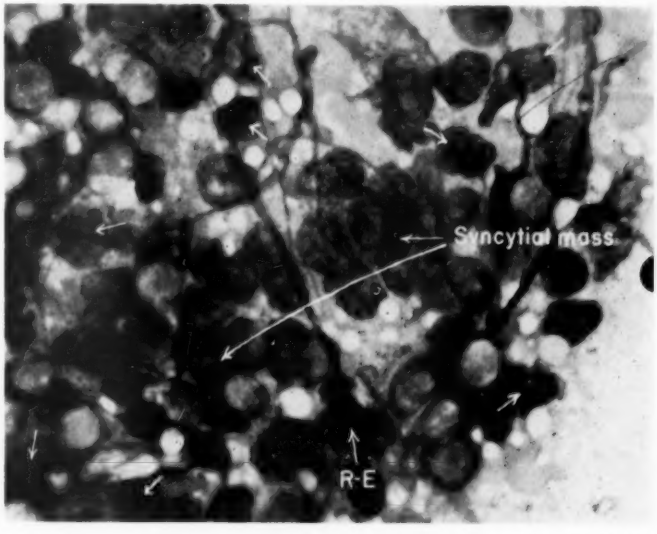

Fig. 7.—Spleen of the rat 24 hours after one injection of carbon tetrachloride. Large syncytial masses of reticuloendothelium obliterating the normal pattern are observed; reduced approximately 15% from mag. ×842.

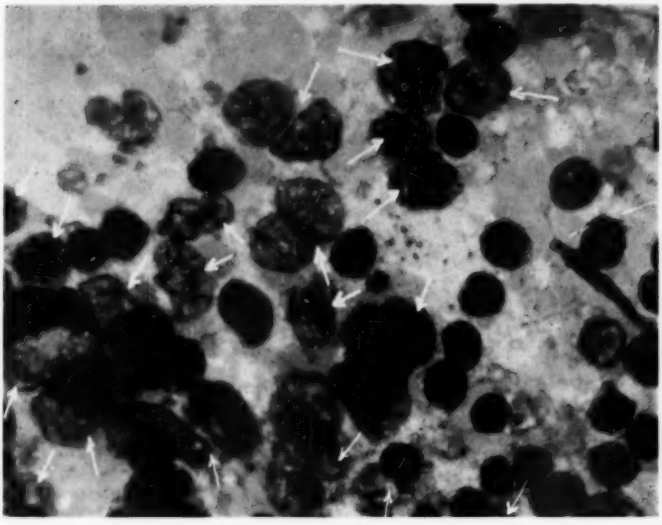

Fig. 8.—Spleen of the rat 48 hours after one injection of carbon tetrachloride. Arrows indicate reticuloendothelial cells. Note marked proliferation in contrast to normal and aberrations of structure. Three plasmacytes are noted lower center; reduced 15% from mag. × 842.

animals. No attempt to enumerate a splenogram was made in animals with syncytia. The other two animals also had a marked increase in these elements, but masses had not formed.

Seventy-two hours after a single injection of carbon tetrachloride, two of four animals again had a marked number of syncytia of cells of reticuloendothelial origin. The two animals without syncytia had a marked elevation of reticuloendothelium, the cells occurring singly.

Spleens observed during the remainder of this study at 120 and 168 hours illustrate a marked number of syncytia with a few free reticuloendothelial cells present. Lymphocytes were relatively decreased, and all semblance to the normal cytologic picture was eradicated. A moderate mast-cell response was noted only at 168 hours (Fig. 9).

A plasmacytosis is noted as early as 24 hours after a single injection. A marked number of plasmacytes are observed at 48 hours, with slowly decreasing levels which do not return to normal levels at any time during this experiment.

After one injection of carbon tetrachloride, a mild increase of neutrophils is noted at 18 and 24 hours, with a mild eosinophilia at 24 hours. Neutrophils and eosinophils remain within 95% confidence limits during the remainder of this experiment in the spleen.

Production of megakaryocytes, metarubricytes, and basophils, which normally occur in minimal numbers in the rat spleen, apparently was unaffected by this experiment, and no alteration in their numbers above 95% confidence limits was noted.

Marrow.—Femur marrow of the normal mature rat reveals a highly cellular content. The majority of these cells fall into neutrophilic, rubricytic, and lymphocytic series. An occasional eosinophil is noted.

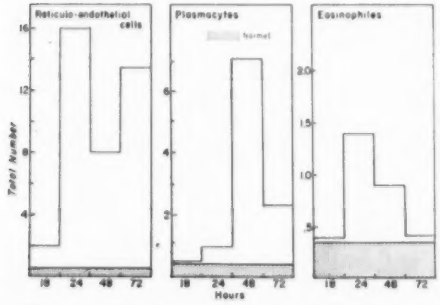

Chart 1.—Splenic population. Comparison of normal values with those found after injection of carbon tetrachloride.

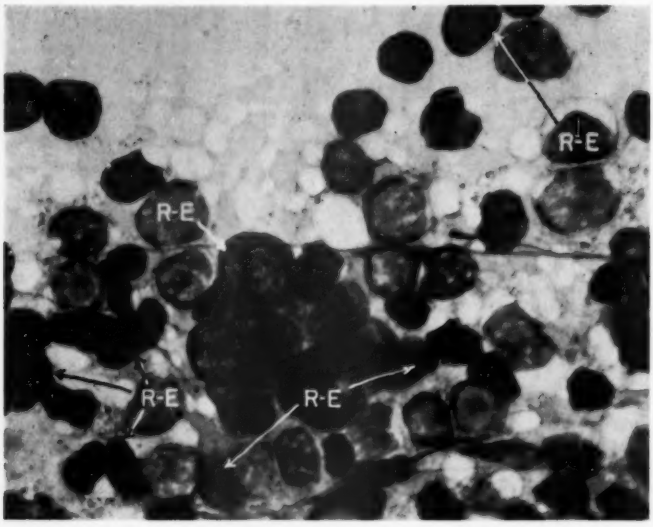

Fig. 9.—Spleen of the rat 120 hours after one injection of carbon tetrachloride. Large syncytial masses of reticuloendothelium; reduced approximately 15% from mag. × 842.

Other normal marrow constituents are relatively sparse, with basophils, monocytes, reticuloendothelial cells, plasmacytes, mitotic cells, and mast cells *in toto* comprising only approximately 6% of the total population. Megakaryocytes are abundantly distributed throughout, with only a rare precursor observed. The normal reticulum cell of the marrow is approximately 15μ with a vesicular chromatin pattern of the nucleus, which occupies the majority of the cell volume. A small nucleolus is usually present, which stains the same intensity as the rest of the nucleus. The cytoplasm is deeply basophilic, as stained with Wright's stain, and lacks granulation. A complete study of the rat marrow will be published in another communication. Normal mean

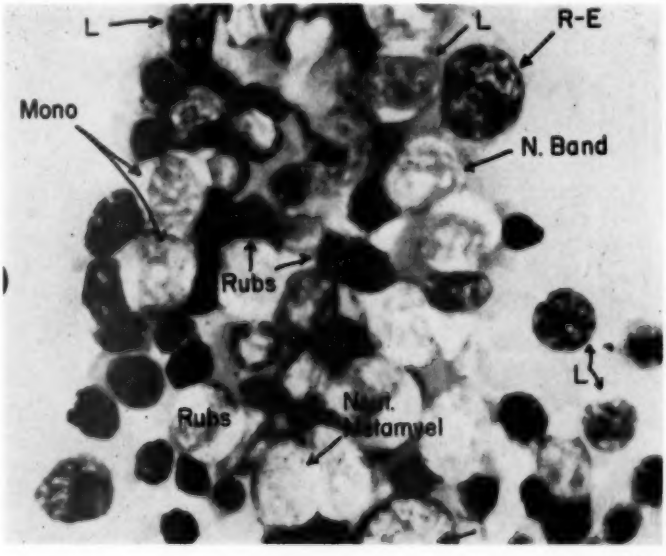

Fig. 10.—Femur marrow of the normal rat. This field was chosen to illustrate morphology of a normal reticuloendothelial cell; reduced approximately 15% from mag. × 842.

TABLE 3.—*Distribution of Femur Marrow Population in Acute CCl_4 Toxicity Mature Long-Evans Rats*

Time, Hr.	Rats, No.	Total No. of Neut.	Eos.	Baso.	Mono.	R-E.	P. C.	L.	Rub.	Mit.	Maste.
18	2	19.6	5.7	0.9	4.3	6.0	1.0	29.5	33.0	0.2	0.0
21	2	21.3	7.2	0.8	2.9	4.9	2.5	20.8	40.5	0.0	0.0
48	4	21.2	12.8	1.9	4.8	4.4	1.7	23.9	29.0	0.0	0.0
72	3	29.0	7.6	0.9	2.6	8.1	2.4	18.9	27.6	0.1	0.0
120	2	28.2	6.9	1.2	2.3	3.2	0.9	20.1	37.2	0.2	0.0
168	2	28.3	10.0	2.4	3.5	1.7	1.3	21.3	31.8	0.0	0.0
Normal	20	25.7	6.2	0.9	0.7	1.3	1.3	36.5	27.3	0.2	0.01

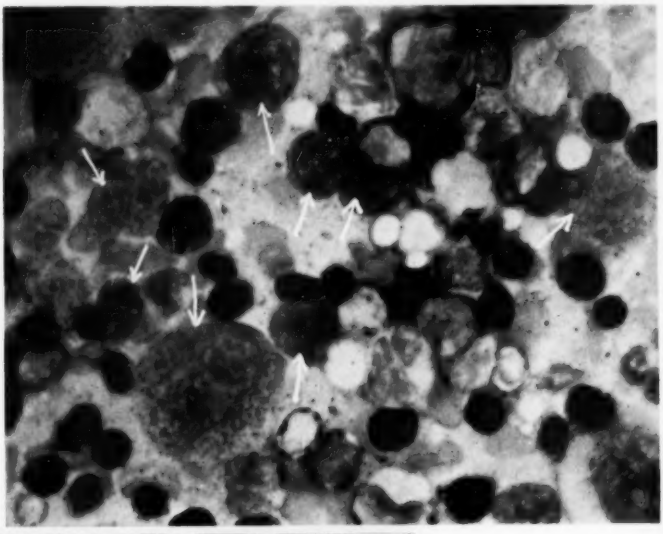

Fig. 11.—Femur marrow of the rat 24 hours after one injection of carbon tetrachloride. Marked proliferation of reticuloendothelial cells. Several have very large prominent blue nucleoli; reduced 15% from mag. × 842.

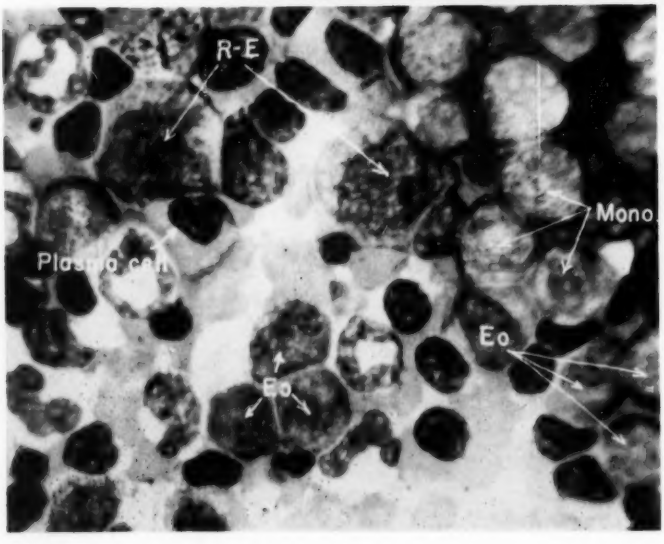

Fig. 12.—Femur marrow of the rat 48 hours after one injection of carbon tetrachloride. Reticuloendothelial cells have vesicular nuclei with multiple prominent nucleoli; reduced 15% from mag. × 842.

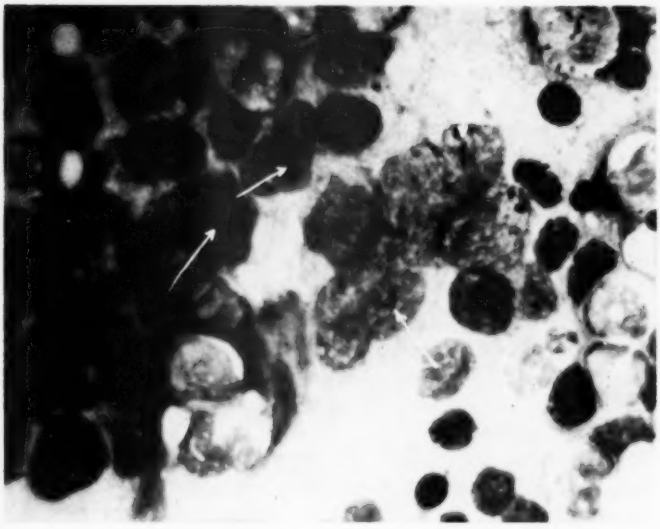

Fig. 13.—Femur marrow of the rat 72 hours after one injection of carbon tetrachloride. Arrows indicate multinucleated reticuloendothelial cells; reduced 15% from mag. × 842.

values are given in the marrow table accompanying this paper (Fig. 10 and Table 3).

After a single injection of carbon tetrachloride, an initial marked increase at 18 hours is observed of cells of reticuloendothelial origin. Many still retain normal morphology, but numerous aberrations are noted. Many are in amitotic division, and other cells of this series are up to 30μ in size, with two to three very large prominent blue nucleoli and a diffuse azurophilic granulation spread over the entire cell volume. This same general picture pervades through 48 hours (Figs. 11 and 12).

At 72 hours, a further increase of cells of reticuloendothelial origin is noted, with numerous aberrations. Many forms, approximately 15μ in size, with deeply basophilic cytoplasm and several small blue nucleoli, are observed. Occasional syncytial masses of these forms occur. Cells mor-

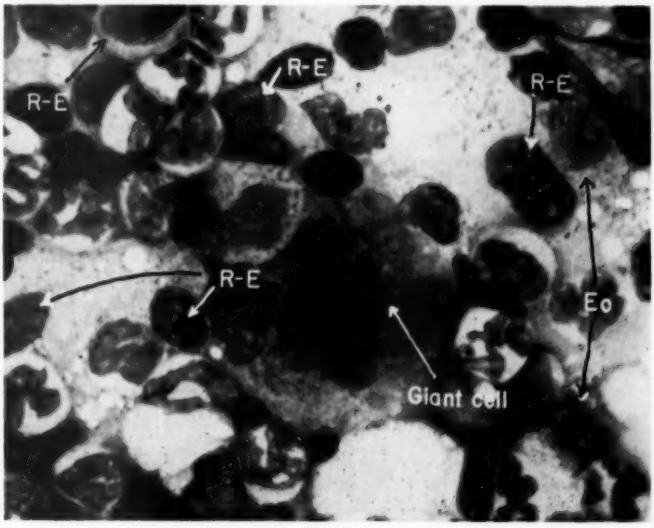

Fig. 14.—Femur marrow of the rat 168 hours after one injection of carbon tetrachloride. A typical giant cell observed in this study is illustrated; reduced approximately 15% from mag. × 842.

phologically similar to those noted in the earlier observations are also present in large numbers. Giant cells were not observed at this interval (Fig. 13).

A quantitative decrease of this series is noted at 120 hours, with a normal value at 168 hours. However, on a cytologic basis, the morphologic criteria are deviated, and it is at this time that giant cells of the foreign-body type make their initial appearance. These cells have 10 to 12 nuclei each, with a vesicular structure and one to two small nucleoli. The cytoplasm is basophilic staining, usually with vacuoles, and may have a few azurophilic granules. Cells with aberrations as noted above still persist at the termination of this experiment at 168 hours (Fig. 14 and Chart 2).

Monocytes are consistently elevated throughout the experiment, with a peak rise initially at 18 hours and again at 48 hours. Eosinophils show a marked elevation at 48 hours, with values returning to near normal, and then another peak at 168 hours after a single injection. Basophils are also significantly increased only at these intervals. Neutrophils remain within normal limits throughout the study.

Plasmacytes are elevated at 24 and 72 hours, with an early return to normal values at 120 hours. A relative decrease of lymphocytes is noted throughout. Nucleated erythrocytes are above 95% confidence limits at 24 hours and again at 120 hours (Chart 2).

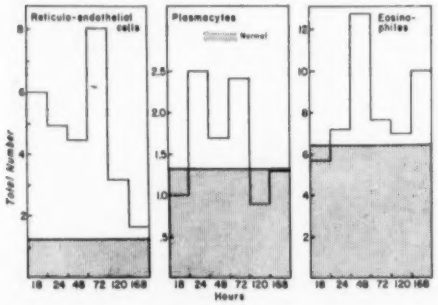

Chart 2.—Femur marrow population. Comparison of normal values with those found after injection of carbon tetrachloride.

Reticulocyte levels in the peripheral blood, however, remain within normal limits throughout this study, without any significant increases. Mitosis does not increase at any time during this study. Amitotic cells were not included in enumeration of mitosis. A mast-cell response was not observed in the marrow as in other organs, although the mast cell is a normal, but infrequent, component of the normal marrow.

Megakaryocytes remained abundant throughout the experiment, without any apparent increase in precursors or morphologic changes.

Comment

This section will now attempt to correlate the hepatic histological changes that were observed during this study with the imprints of cellular morphology of the hemopoietic system.

Liver Imprints.—Most interestingly, the cytological characteristics of the liver imprints obtained from the experimental animal did not deviate from the normal, although the histological changes were marked. Even in the presence of marked diffuse infiltration of inflammatory cells and necrosis of hepatic cells that extended to the periportal zone at 48 hours after one injection of carbon tetrachloride the liver imprints did not show any differentiation from the normal.

It would seem to us that such absence of correlation in the liver imprints rather indicates that the techniques for liver imprints in experimental cirrhosis are still limited or lacking. Why do we say that?

Bone Marrow Imprints.—Because the bone marrow at 18 hours, after a single injection of carbon tetrachloride subcutaneously, produces a prompt marked initial increase of the cells of reticuloendothelial origin. Numerous aberrations may be noted. The changes persist through 48 hours; at 72 hours a further increase of reticuloendothelial cells is noted, with similar aberrations of the type previously observed. Although a quantitative decrease of this series then occurs, returning to nor-

mal values at 168 hours, a morphological deviation takes place at this point. Giant cells of the foreign-body type make their appearance. Monocytes, eosinophils, and basophils all increase throughout the experiment; neutrophils alone remain constantly normal. Plasmacytes too show an elevation, as do the nucleated erythrocytes, and then, in contrast, the lymphocytes decrease.

Spleen Imprint.—The involvement of the liver in this classic carbon tetrachloride picture at 18 hours is evidenced in every liver section by a moderate degree of central necrosis, swelling, and disruption of the cytoplasm. At this interval, there are only relative changes in the spleen. At 24 hours, the spleen shows a marked augmentation of reticuloendothelial cells and eosinophilic series, and a slight plasmacytosis occurs. The splenic imprints reach the highest peak of plasmacytosis at 48 hours, and it is at this time that the liver section shows the more diffuse infiltration of inflammatory cells and the greatest degree of necrosis.

One hundred twenty-eight and one hundred sixty-eight hours after injection of carbon tetrachloride, after the liver is greatly recovered and is returned to its normal architecture, the spleen still shows marked syncytial masses of reticuloendothelial cells. The same finding is encountered in the femur marrow. After the liver sections appear healed, evidence of reaction still persists in the hemopoietic system.

This profound reaction of the reticuloendothelial system, unsuspected until now, indicates an important need to evaluate the impact of a particular poison or chemical not only on the kidneys and liver but on the hemopoietic system in general and the cells of reticuloendothelial origin in particular. The very fact that the reticuloendothelial system in this instant is still reacting to the single injection of carbon tetrachloride, when the liver and the kidneys have already been restored to normal architecture, is an index to the sensitivity of this particular system of cells.

Summary

Evidence is presented as to the reticuloendothelial system involvement in animals given a single injection of carbon tetrachloride.

Lymph Node.—The reticuloendothelial response is marked at 48 hours, with considerable deviation from normal morphology. A decrease in numbers of this series occurs at later observations, with complete disappearance of pathologic forms. A plasmacytosis is evident in the lymph node throughout the study.

Thymus.—Reticuloendothelial cells are not increased in the thymus. A plasmacytosis is observed at 48 hours, which reaches a peak at 72 hours and then gradually diminishes, with only an occasional plasmacyte remaining at 168 hours.

Blood.—An early mild neutrophilia with a more marked monocytosis is present in the initial observations. However, from 48 through 120 hours, a neutropenia with a concomitant eosinophilia is noted. All series are within normal confidence limits at 168 hours.

Liver.—Liver imprints do not deviate from the normal cytological characteristics in this study.

Spleen.—An increase of cells of reticuloendothelial origin is noted even at the 18-hour observations. As early as 48 hours, large numbers of syncytial masses of reticuloendothelial cells are present. This series remains markedly increased throughout the study. Deviations from normal morphology are similar to those noted in the lymph node and other organs. A plasmacytosis is also evident in the spleen through 168 hours.

Marrow.—The initial prominence of cells of reticuloendothelial origin is observed, with deviations of morphology similar to those seen in other organs. Numerically,

this increase reaches a peak at 72 hours. However, cytologically, the most interesting forms are revealed at the termination of this study with the presence of foreign-body type giant cells and other distinct aberrations of this series. In the early phases of the experiment, monocytes, eosinophils, nucleated erythrocytes, basophils, and plasmacytes are increased.

Mr. Dean C. Altman, head of the Illustrations Department of the Veterans' Administration Hospital, prepared the photomicrographs.

Veterans' Administration Hospital, Sam Jackson Park (7).

The Effects of Cortisone upon Aortic Intimal Repair in the Hypercholesteremic Rabbit

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The present investigation is a continuation of studies carried out over a period of several years in this laboratory designed to shed light upon the pathogenesis of atherosclerosis (Table). These studies, which have concentrated upon the reparative process following intimal injury in the experimental animal, were stimulated by several observations. The first of these was the consistent finding of microscopic fibroelastic "plaques" in the aorta of all children over the age of 2 weeks.[1] Since these lesions were nearly devoid of sudanophilic material, it was suggested that they might represent the earliest phase in the development of the adult plaque, and the question was raised whether they might be a manifestation of intimal repair. Another impetus for this investigation of intimal repair was the reemphasis of Rokitansky's[2] hypothesis by English investigators[3-7] that lesions identical morphologically with atherosclerosis could be produced by the organization of arterial thrombi.

Studies on the Pathogenesis of Atherosclerosis

1. Repair of aortic intimal mechanical trauma in rabbits.
2. Effects of hypercholesteremia on the rabbit aorta.
3. Repair of aortic intimal trauma in hypercholesteremic rabbits
4. Effects of cortisone upon rabbit blood vessels.
5. Effects of cortisone+cholesterol on the rabbit aorta.
6. Effects of cortisone upon repair of rabbit aortic intimal trauma.
7. Effects of cortisone+cholesterol upon rabbit aortic intimal trauma.

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Departments of Pathology and Surgery, State University of New York Upstate Medical Center.

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We have previously demonstrated that mechanically induced aortic intimal injury in the rabbit resulted in the production of a localized fibroelastic thickening within which minute amounts of lipid could be demonstrated after periods of five months.[8] These "plaques" showed little similarity to the lesions of human atherosclerosis but were histologically inseparable from the fibroelastic thickenings noted in infants. As a result of this observation studies were carried out to elucidate the reparative process in the hypercholesteremic rabbit following mechanical intimal trauma.[9] The injured vessel segment in these animals showed increased permeability to cholesterol, and 14 days after injury a plaque composed of lipid material and connective tissue was noted. Although at this stage the lesion was histologically identical with human plaques, its subsequent course was characterized by necrosis and inhibition of connective tissue repair. Forty-six days after injury the traumatically induced lesions were structurally similar to the spontaneously arising hypercholesteremic plaques. We suggested that this inhibitory effect of cholesterol upon connective tissue repair might explain why the histologic counterpart of the advanced human atherosclerotic lesion cannot be duplicated experimentally in the rabbit by cholesterol administration.

Although the origin and manner of deposition of the lipid component of the atherosclerotic plaque remains highly controversial at present, there seems to be general agreement that the subintimal tissue of a plaque does show early evidence of connective tissue injury and subsequent reparative activity. In an effort to more clearly define the role

of fibroplasia in the genesis of the atherosclerotic plaque, we elected to study the progression of experimental atherosclerosis under the influence of cortisone. In addition to its known suppressive effect upon repair, it was hoped that its influence upon the inflammatory process might also be helpful in adding to our knowledge concerning the localization of lipid in experimentally produced plaques.

Methods

The present study, which concentrated upon the effects of cortisone upon intimal repair in the hypercholesteremic rabbit, necessitated a background of data based upon six other experimental groups (Table).

Group 1, a study of aortic intimal trauma, has been summarized above and was the subject of a previous publication from this laboratory.[8] Groups 2 and 3 have been referred to above and have also been previously reported.[9] The present study will then record observations of Groups 4-7.

All animals used were New Zealand White rabbits, and they were approximately evenly divided as to sex. Their age at the beginning of the experiment was 8-10 weeks, and their average weight was 2200 gm. They were housed separately in wire cages and fed Purina Rabbit Chow or chow supplemented with 1 gm. of cholesterol per day. The latter was administered as the purified substance dissolved in ether, mixed with food pellets, and allowed to evaporate. The cholesterol dietary supplement was instituted the same day the intimal trauma was carried out. Blood cholesterol values were determined at the time of killing of cholesterol-fed animals. These indicated early and rather marked elevation of cholesterol, but we did not feel that the cortisone administration was responsible for unusually high elevations. The cortisone* preparation used throughout the experiment was cortisone acetate in a sterile aqueous suspension and was administered intramuscularly as a single daily injection.

The aortic intimal trauma was carried out under thiopental (Pentothal) and ether anesthesia, and the peritoneal cavity was entered under sterile precautions. The abdominal viscera were retracted from a segment of lumbar aorta, and the latter was freed from the adjacent connective tissue with a minimum of trauma. The aorta was then entered at an oblique angle with a 24-gauge needle, the point of which was deliberately angulated to effect a severer tissue injury. The aortic intimal surface was then traumatized by repeated vertical and horizontal motions. Although this technique did not permit control of the depth of the injury, we were in general more interested in those areas in which the injury was superficial, i. e., intima and inner media. The needle was withdrawn and hemostasis effected by means of direct pressure over the puncture site. Before the peritoneal cavity was closed, the traumatized area was marked by means of nonabsorbable (silk) surgical sutures placed in the periaortic connective tissues.

The animals were killed at intervals ranging from 1 day up to 12 weeks after the beginning of the experiment. The entire aorta was removed, and areas remote from the traumatized zone were considered to serve as controls. The cortisone acetate was administered intramuscularly in the thigh in dosages ranging from 0.1 mg. per rabbit up to 15 mg. per rabbit daily. Because of the susceptibility of those animals on high cortisone dosages to pulmonary complications, some animals were given penicillin G, U. S. P., biweekly. Cortisone administration was instituted either concurrently with the date of aortic injury or a few days before.

Pathologic Findings

Group 4 (Effects of Cortisone Alone).—Six animals were maintained on 15 mg. per rabbit daily of cortisone acetate and were killed at intervals of from 8 days to 12 weeks. A variety of tissues were studied at autopsy, but there was particular emphasis placed upon the vascular system. Effective levels of cortisone were present early, as evidenced by the fact that there were multiple areas of autolytic hepatic necrosis (somewhat similar to changes seen in human fulminating hepatitis) as early as 18 days after commencement of the drug (Fig. 1). The liver also showed diffuse fatty changes, and striated muscles in a variety of locations showed necrosis and calcification. In animals maintained on this cortisone dosage for two months there were striking pulmonary changes, consisting of acute bronchitis and bronchiolitis, bronchopneumonia, and abscess formation. The penicillin G preparation was not particularly effective in prolonging the life span of those animals in which pulmonary complications developed. In these animals killed at two months the striated muscle atrophy and calcification was

* Cortisone acetate was supplied by Dr. H. C. Peltier of The Upjohn Company, Kalamazoo, Mich.

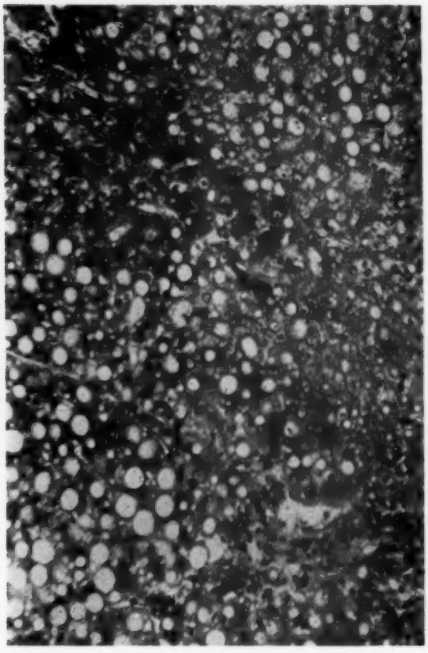

Fig. 1.—Severe fatty changes throughout liver, with autolytic necrosis in right half of illustration; × 133.

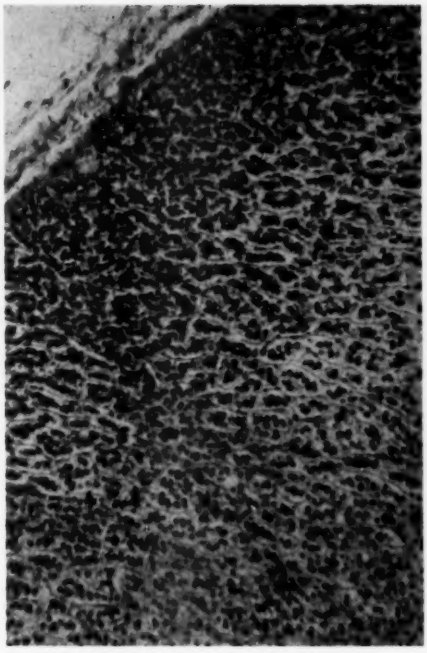

Fig. 2.—Low-power view of adrenal cortical structure from a normal control; × 133.

pronounced and there was also evidence of focal degeneration and calcification of myocardial fibers. Kidney abscesses, chiefly cortical in location, and calcification of renal tubular epithelium and basement membrane were prominent. The adrenal glands which showed no significant changes at one month were characterized by swelling and degeneration of the cells in the inner zone fasciculata at two months (Figs. 2 and 3). We could see no evidence that cortisone at this dosage level had any effects on either the aorta or the visceral vessels.

GROUP 5 (Effects of Cortisone Plus Cholesterol).—Eight animals were maintained on 15 mg. per rabbit of cortisone and were killed at intervals of from 9 days up to 2 months. These animals, which were also receiving 1 gm. of oral cholesterol per day, showed no evidence of either gross or microscopic atherosclerotic changes. This finding seems particularly significant by reason of the fact that we have previously observed microscopic plaques 27 days after this same cholesterol dosage and that abundant gross lesions were present on the aortic intimal surface as early as 46 days on a similar cholesterol dosage.[9] In addition, examination of other tissues failed to show evidence of cholesterol deposition in other areas (spleen, skin, liver, adrenals), a characteristic finding in cholesterol-fed rabbits. The changes in the lungs, liver, adrenal glands, kidneys, and myocardium were similar to those described in Group 4 above. Finally, we could see nothing to suggest that cholesterol administration in any way affected the time of onset or the severity of these visceral lesions.

GROUP 6 (Effects of Cortisone upon Aortic Injury and Repair).—Thirteen animals were subjected to aortic injury as outlined above and were administered 15 mg. per rabbit daily of cortisone, the latter beginning the day of or a few days before the date of the aortic injury. The animals were killed at intervals of from 3 days up to

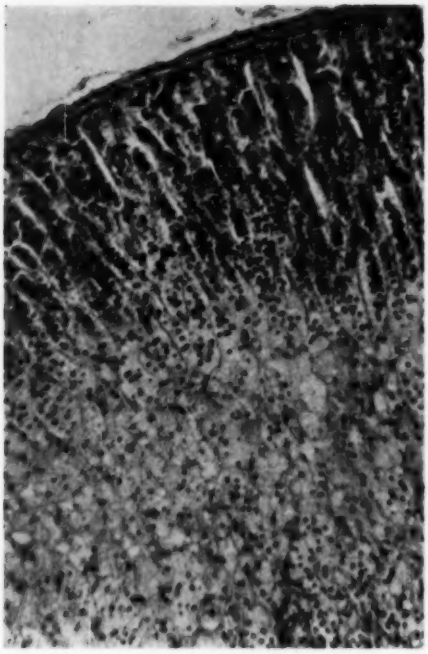

Fig. 3.—Adrenal cortical degeneration in animal maintained on 15 mg. of cortisone for two months. This is the same magnification as Figure 2; × 133.

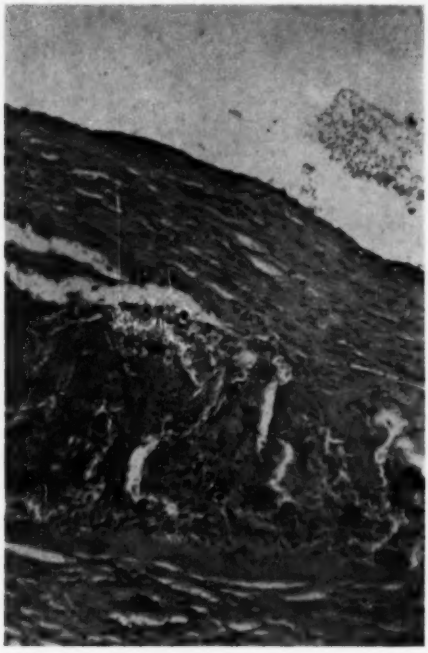

Fig. 4.—Organization of intramural hematoma in traumatized area of cortisone-injected animal 17 days after injury; × 119.

2 months after the date of vessel injury. Very little regenerative activity was observed in the first 10 days after the trauma. Up until that time the microscopic appearance of the injured segment was characterized by either broad zones of fibrinoid necrosis or intramural hemorrhage. Minimal attempted covering of the endothelial surface was noted, and there was practically no inflammatory reaction about the margins of the fibrinoid and hemorrhagic areas. Rare mononuclear cells could be identified, but they were more prominent in the periadventitial connective tissues, where they were seen accumulating about necrotic fatty foci. Some giant cells were observed in these areas. Seventeen days after injury the endothelial defect, although depressed, was covered by a layer of large bizarre-shaped endothelial cells. The intramural hemorrhage showed invasion by granulation tissue (Fig. 4). Healing, which was completed by two months, was characterized by a dense collagenous scar tissue covered by a layer of mature endothelial cells (Fig. 5). Considerable periadventitial acute and chronic inflammatory reaction persisted at this time.

Group 7 (Effects of Cortisone Plus Cholesterol upon Aortic Injury and Repair).—Forty-six animals were studied in this group. The oral cholesterol supplement was instituted on the same day as the aortic trauma was carried out, but the intramuscular cortisone was begun a few days before this date in the majority of the animals. The cortisone dosage ranged from 0.1 mg. per rabbit up to 15 mg. per rabbit daily, and the animals were killed at periods of from 3 days up to 12 weeks after the aortic injury. Three days after injury the depressed intimal segment was represented by a homogeneous pink band of fibrin and edema fluid. Rare mononuclear cells with

Fig. 5.—Healing of the aortic injury in a cortisone-injected animal two months after injury; × 119.

Fig. 6.—Fibrinoid edematous appearance of the injured area five days after injury in an animal given cortisone plus cholesterol; × 119.

a gray granular cytoplasm were seen, and these tended to collect about the margins of the zone of fibrin. Aggregates of polymorphonuclear leukocytes were noted in the periadventitial tissue, and these appeared to be a response to the injured fatty tissue. Five days after injury the mononuclear cells were slightly more numerous and occasional cells had the pale slightly foamy appearance of the so-called lipophages (Fig. 6). Acute inflammatory cells had collected about the injured perivascular fat, and many of these showed degenerative changes. The injured intramural tissue was fibrinoid in appearance at this time. Endothelization of the smaller denuded intimal areas was completed in five to six days (Fig. 7). Eight days after injury there was invasion of the fibrinoid zone by a relatively nonvascular connective tissue and no significant increase in the number of histiocytes was observed. The adventitial reaction was less marked and appeared to be essentially chronic in nature at this time. Examination of sections taken one month after injury showed progressive fibroblastic proliferation about the hyalinized intramural tissue. Some new capillaries were present, but much of the hyaline material persisted (Fig. 8). The former injured endothelial surface was capped by a fibrous layer of considerable thickness, and the endothelial cell covering seemed mature in type. Inflammatory cells were not present within the vessel wall, although mononuclear cells did persist in the foci of perivascular fat necrosis. No evidence of fat-filled macrophages was noted in the vessel itself. Finally, it is pertinent to point out that the changes just recounted were characteristic of those animals on high cortisone dosages, i. e., 15 mg. per rabbit. Dosages of cortisone below this level tended to show the changes characteristic of trauma and cholesterol alone. While there was no gross or microscopic evidence of spontaneous atherosclerosis in the animals on higher

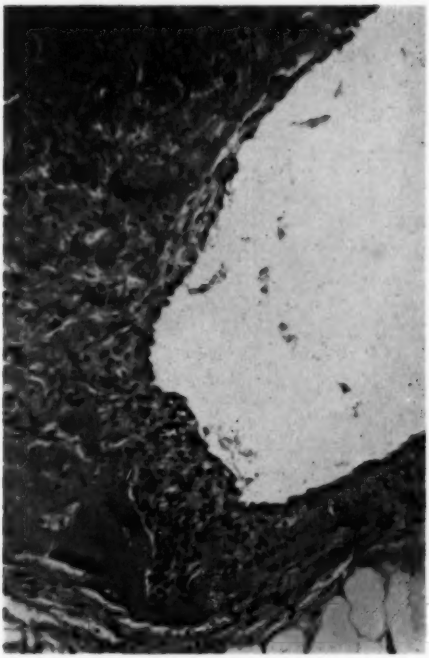

Fig. 7.—Endothelization of the injured segment in an animal given cortisone plus cholesterol nine days after injury; × 140.

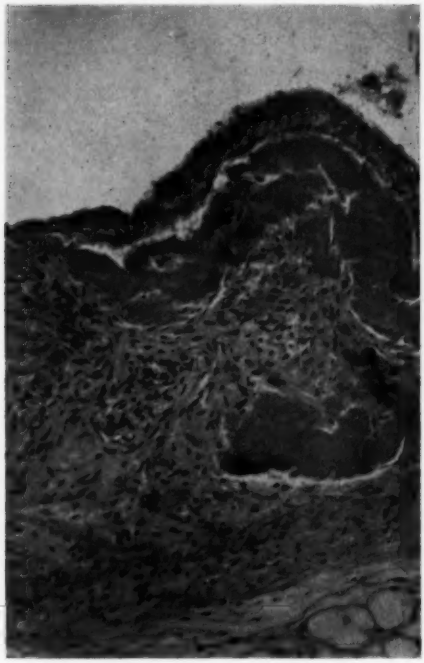

Fig. 8.—Healed phase one month after injury in an animal given cortisone plus cholesterol. Dense hyaline material persists; × 140.

cortisone dosage even at 12 weeks, there was abundant intimal fatty change throughout the intima in the animals on lower cortisone dosages.

Comment

Several studies are in agreement regarding the fact that cortisone is capable of inhibiting experimental atherosclerosis in the rabbit, although there is some difference of opinion regarding the serum lipid levels in these animals and the possible mechanism of action exerted by cortisone. Oppenheim and Bruger[10] injected 10 mg. of cortisone into five rabbits three times weekly for 10 weeks. They noted no effect on the serum cholesterol level, but they did observe a definite increase in the serum phospholipids. Cortisone inhibited the formation of aortic atherosclerosis in their cholesterol-fed rabbits, even though the hypercholesteremia was accentuated and the phospholipid-cholesterol ratio was markedly depressed. They stated that corticotropin (ACTH) was also antiatherogenic but not to the same degree as cortisone. Gordon and co-workers[11] administered cortisone to cholesterol-fed rabbits over a three-week period in dosages ranging from 1 mg. per kilogram per day to 3 mg. per kilogram per day. Although they observed less atherosclerosis in these animals than in cholesterol-controls, they did state that cortisone depressed appreciably the hypercholesteremia resulting from feeding cholesterol to rabbits. They also described a moderate increase in the serum lipid phosphorus, a great increase in the serum neutral fat, and a doubling of the cholesterol-lipid phosphorus ratio in the cortisone-plus-cholesterol group. Stumpf and Wilens,[12] however, made the point that lipid deposition within the vessel wall may be at variance with the degree of the hypercholesteremia attained. They felt that this may be because other lipid components, i. e., phospholipids, are increased. They

noted that the lipid deposition was less than one-half as extensive in the aortas of six rabbits receiving cortisone-plus-cholesterol as in six that were fed cholesterol without cortisone. The cortisone dosages ranged from 5 to 15 mg. per animal per day, and the duration of this study was 84 days. They stated that cortisone had no effect on the cholesterol-phospholipid ratio, and they pointed out that the conditions that modify experimental atherosclerosis may not exert the same effect in man. Unfortunately there is little evidence either to support or to deny this statement. A poorly documented unconvincing study by Etheridge and Hoch-Ligeti [13] described the aortas of 33 patients under 21 years of age who had been treated with cortisone and/or corticotropin. They concluded that there was increased lipid deposition in both the intima and the media in the patients under age 11 who received cortisone or corticotropin. After this age lipid was found so frequently that comparison between the two groups was equivocal. There appeared to be no correlation between the degree of atherosclerotic change and the amount of hormone received.

The effects of cortisone upon the vascular system have been studied in other animals. Paterson and Mitchell [14] investigated the effects of cortisone (4.5 mg. per kilogram of body weight) over a 15-day period upon "spontaneous" coronary arteriosclerosis in chickens. They concluded that cortisone brought about both a reduction in the number and in the size of coronary intimal plaques. They alleged that it was due to the known fibrolytic activity of cortisone but stressed the fact that the lesions in chickens were nonlipid and essentially fibrous tissue in composition. Some studies have been carried out regarding the effects of cortisone on aortic healing in dogs, with particular reference to vessel grafts. Barberio et al.[15] placed fresh and frozen homografts in dogs for periods up to 21 days. Cortisone was administered to the animals (3 mg. per kilogram) for 14 days postoperatively. It was hoped that cortisone would prevent thrombosis and excess inflammatory changes at the graft sites. They observed a marked decrease in fibroblastic proliferation subendothelially and at the suture lines. Endothelization from the adjoining recipient aorta was estimated to be comparable to that seen in the control group. A decrease in the degree of phagocytosis was apparent in the cortisone-treated group. They concluded that cortisone had no effect on the frequency or extent of thrombosis at the graft sites. Similarly, Kroboth and co-workers [16] found that cortisone did not impair the healing of homografts in dogs or effect the incidence of thrombosis in the grafts. They did feel, however, that the size of the thrombus formed was greater in dogs that had received cortisone.

There is no agreement regarding the mechanisms by which cortisone inhibits experimental cholesterol atherosclerosis. Gordon and co-workers [11] postulated several possible mechanisms. They suggested that the inhibition of atherosclerosis might be due to alterations in serum macromolecular lipoproteins, with a resultant change in the aortic endothelial-cell permeability. Studies utilizing the ultracentrifuge [17] have suggested that prolonged treatment with cortisone may cause a metabolic block at the level of S_f80 in the breakdown of higher S_f series to the lower ones. The suggestion is advanced that higher lipoproteins are not as atherogenic as lower ones. Adlersberg et al.[18] have studied the effects of corticosteroids on tissue permeability in rabbits. They demonstrated that the additional elevation of plasma cholesterol and the inhibition of atherogenesis produced by cortisone administration in cholesterol-fed rabbits were counteracted by the simultaneous administration of hyaluronidase. They stated that the retardation of atherogenesis in cortisone-treated cholesterol-fed animals could be explained by the diminished tissue permeability to lipids. Our studies have lead us to concentrate upon the role of the histiocyte in the inhibition of atherosclerosis within the cortisone-treated group. We have shown a striking diminution in the number of intramural lipophages at any stage of the experi-

mental lesion. Although the origin and the role of the histiocyte in the genesis of the atherosclerotic plaque is not settled, there can be no doubt that it plays a very important role, even in the earliest stages of the evolution of the plaque. Studies relating to the effects of cortical hormones on experimental infection may be quite pertinent to our hypothesis. It has been shown that cortisone alters the defense mechanisms by its effect on the fixed macrophages of the reticuloendothelial system. Robinson and Smith [19] noted that when pneumococci were injected intravenously into normal rabbits the bacterial cells were rapidly cleared from the blood and that the animals survived the infection. When corticotropin- or cortisone-treated animals were given a similar intravenous injection the rate of disappearance of the pneumococci from the blood was similar to that of normal rabbits during the first few hours but thereafter the number of bacterial cells rapidly increased and overwhelming sepsis occurred. These findings suggest, among other things, that the ability of the fixed macrophages to destroy virulent pneumococci is altered in some manner by cortisone administration. Moss and Drury [20] have also commented upon the collapse of foam cells within the intima of cholesterol-fed rabbits receiving cortisone. The plaques in these animals, when present, contained only a small number of foam cells, while the major portion of the plaque was composed of fibroblasts, delicate connective tissue fibers, a few lymphocytes, and some neutrophilic leukocytes. The blood cholesterol, however, in these animals was three times greater than that in rabbits fed high cholesterol but not given injections of cortisone.

Summary

A study designed to investigate the effects of cortisone upon intimal injury repair in normal and hypercholesteremic rabbits is presented. The daily administration of cortisone at a dosage of 15 mg. per animal prevented the formation of gross and microscopic aortic plaques in hypercholesteremic rabbits which survived two months. Cortisone in this dosage in normal rabbits resulted in delayed and atypical inflammatory and reparative reactions within the injured aortic segment. Repair of the traumatized aortic area in hypercholesteremic cortisone-injected animals was characterized by similar atypical changes and a very striking lack of lipophages. It was suggested that this lack of histiocytic elements in the cortisone-injected animals might explain the observed retardation of atherogenesis. This inhibitory effect of cortisone upon experimental atherosclerosis was observed only in those animals maintained on 15 mg. per animal per day, a level at which the serious lesions in other organs prevented survival for periods of over two months.

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The Role of the Microfollicle in Non-Neoplastic Noninfectious Goiter

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Many histologic and clinical variants of goiter which are not neoplastic and not infectious result from a balance of the number and activity of two distinct morphologic and functional epithelial units: microfollicles and macrofollicles. Each follicular unit has a characteristic pattern of hyperplasia and involution. The activities of the macrofollicle and its metabolic relationship to iodine are well established. However, the pattern of hyperplasia and involution of the microfollicle is not appreciated. Furthermore, the nature of the metabolic stimulus for microfollicular hyperplasia and involution remains obscure. Histologically the vast majority of goiters are not pure but are composed of a mixture of macro- and microfollicular elements. In this country, review of pathologic material shows that the macrofollicle is by far the dominant unit.

McClintock and Wright,[10] Parmley and Hellwig,[11] Goldberg and Davson,[3] and Lindsay et al.[8] have recorded the microfollicular prominence in Hashimoto's disease. Furthermore, Parmley and Hellwig[11] measured the lymphoid and epithelial elements in paraffin sections of Hashimoto's disease of the thyroid gland and determined that only one-third of the enlargement was due to lymphoid proliferation and that two-thirds of the gland was composed of epithelial structures. Statland et al.[13] roughly estimated that the microscopic volume of acinar elements in struma lymphomatosa was slightly more than one-half the total gland. When the over-all gross glandular enlargement, as indicated by the size and weight above normal, is considered with these estimates, an underlying state of microacinar hyperplasia becomes apparent. Thus, Hashimoto's disease seems to offer a source of material for study of microfollicular activity.

After careful review of 143 cases of thyroiditis, 80 patients with acceptable pathologic criteria of Hashimoto's disease were selected for study. Although two distinct histologic structures (microfollicles and macrofollicles) were found in all cases, the microfollicular variant was by far the dominant element. Microfollicular hyperplasia was accomplished by budding of parent follicles or, on occasion, by hollowing out of interfollicular cell nests. Actually, microfollicular hyperplasia by budding can best be seen in the so-called fetal adenoma of the thyroid gland. In our cases the microacini were compact, small, round, structures 30μ to 100μ in diameter. They contained a minute amount of pale pink or faintly basophilic colloid or no colloid. The lining acinar cells were usually tall-cuboidal or low-columnar. Large basal oval nuclei parallel to the long axis of the cell, with diffuse powdery chromatin and one to four nucleoli, were seen. Occasional small single or multiple vacuoles or colloid droplets were present in the abundant eosinophilic cytoplasm. There was no evidence of mitotic activity, no attempt at cellular stratification, and no papillary infolding. However, there was a definite tendency toward intra-acinar epithelial desquamation. On occasion, groups of these desquamated epithelial cells clustered about colloid resembled a multinucleated foreign-body type of giant cell.

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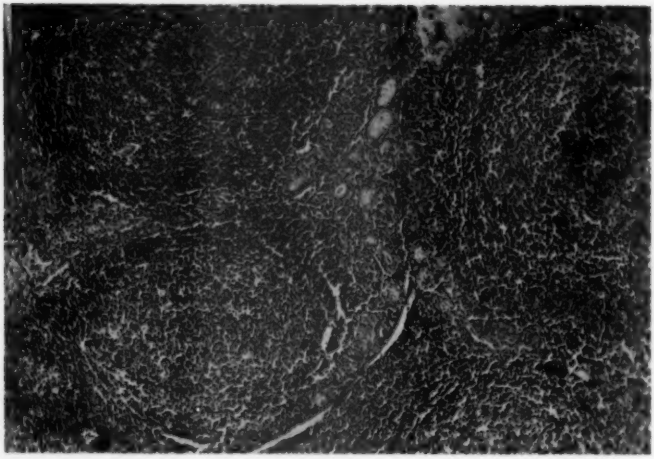

Fig. 1 (Group A).—Multiple large follicles with active germinal centers in microfollicular hyperplastic goiter; reduced 40% from mag. × 120.

The height of these acinar cells—well above normal resting limits—was morphologic verification of cellular hypertrophy. Because of their suspected functional inadequacies, rather than their morphologic appearance, these large acinar cells with frequent Hurthle-like cytoplasmic alteration are customarily mislabeled atrophic.

The intimate association of lymphoid follicle formation, lymphocytic infiltration, intralobular fibrosis, and "giant-cell" formation with microfollicular hyperplasia suggested a process of spontaneous involution.

This progress of spontaneous lymphoid replacement (involution) of microfollicular hyperplasia could be schematically represented as follows:

Group A: Interlobular septal thickening;
lymph nodule formation;
no, focal, or diffuse acinar-cell metaplasia

Group B: Stromal lymphocytic and plasma-cell infiltration

Group C: Degenerative epithelial cell changes;
endothelial hypertrophy and hyperplasia of stromal capillaries and arterioles;
peripheral hyalinization of reticular fibers and capillary walls of stroma

Group D: Reduction in number of acini;
reduction in number of lymphoid follicles;
reduction of lymphocytic infiltration;
diffuse perilobular fibrosis

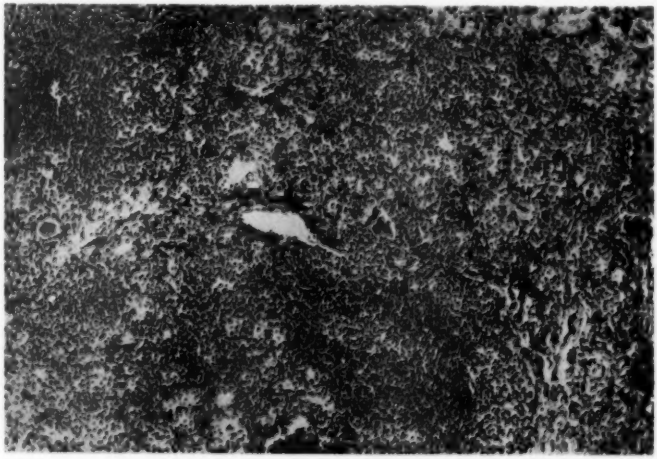

Fig. 2 (Group B).—Active lymphoid follicles with diffuse stromal infiltration by inflammatory cells in microfollicular hyperplastic goiter; reduced 40% from mag. × 120.

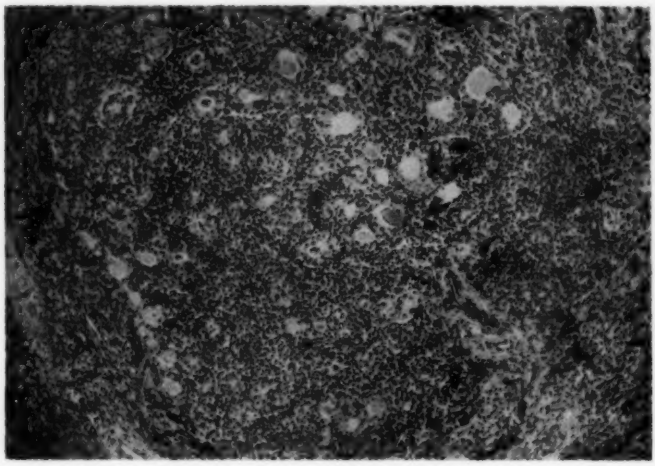

Fig. 3 (Group C).—Diffuse inflammatory cell infiltration of stroma. Marked pleomorphism of hypertrophied epithelium of microacini; reduced 40% from mag. × 120.

Our cases were divided into these four major groups, which appeared to represent chronological progress of involution.

With acinar hyperplasia, there was progressive thickening of the interlobular septa, an exaggeration of the normal trabeculae. The major arteries showed no characteristic alterations. Initially the lymphoid follicles tended to occur immediately beneath the capsule or beneath thickened trabeculae which subdivided the gland into lobules. As the lymphoid follicles grew, they developed active germinal centers. The adjacent acinar parenchyma virtually melted in the advancing margin of these expanding lymphoid masses without creating any visible mesenchymal reaction. The acinar epithelial cell did or did not show focal or diffuse Hurthle-like alteration. The cytoplasm of these altered cells was deeply eosinophilic and increased in amount. The nuclei remained regular, oval or rounded, and basal. Macrofollicles as well as microfollicles were involved in glands which showed diffuse eosinophilic epithelial metaplasia. On the other hand, this type of epithelial cytoplasmic alteration may involve a single cell in a hyperplastic acinus of either variant.

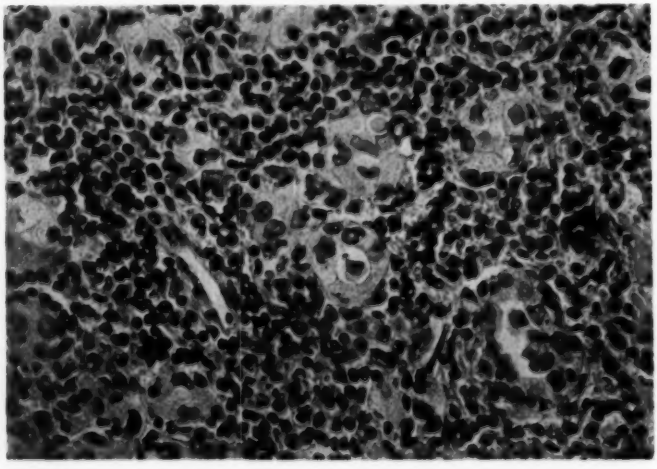

Fig. 4.—Same as Figure 3; reduced 40% from mag. × 600.

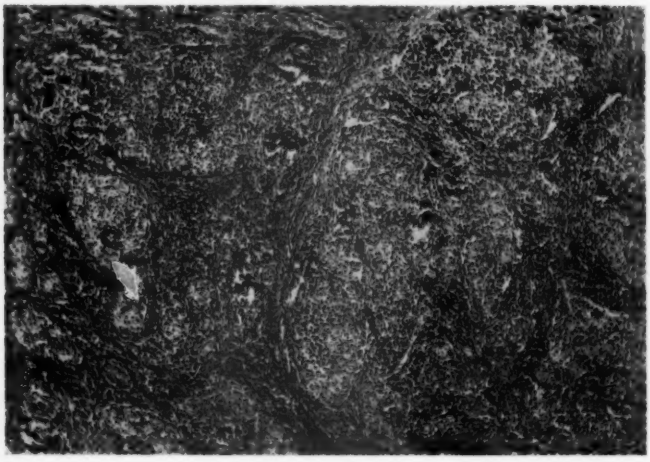

Fig. 5 (Group D).—Early fibrosis, multiacinar "cirrhosis" involving microacinar hyperplastic goiter; reduced 40% from mag. × 120.

Up to this stage, the progress of spontaneous involution, to a large extent, appeared reversible. This capability would account for Hashimoto's original [7] and other occasional reports of spontaneous regression or regression after partial resection of known struma lymphomatosa without subsequent permanent hypothyroidism.

The irreversible steps in the development of spontaneous involution seemed to be initiated by stromal infiltration by lymphocytes and few plasma cells. As this cellular infiltrate became more dense, the endothelial cells of the stromal capillaries and small arterioles showed marked swelling and hyperplasia, with reduction in the caliber of the lumens. Degenerative changes in the acinar cells were indicated by cytoplasmic swelling, pallor, and accumulation of multiple basal vacuoles. The nuclei tended to migrate to the midzone or apex of the cell and were large, pleomorphic, and hyperchromatic. Nuclear long axes, having lost normal polarity, pointed in all directions. As the epithelial cells separated inferiorly and laterally, lymphocytes, macrophages, and plasma cells slipped between them into the colloid. Associated with these vascular and epithelial changes there was intralobular fibrosis. This process of scarring began at

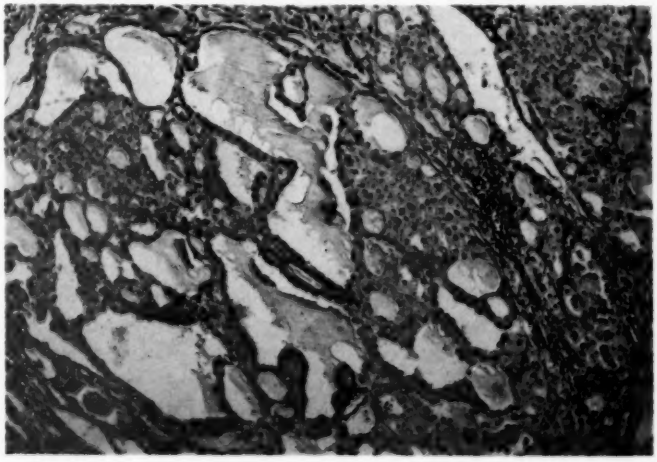

Fig. 6.—Foci of macrofollicles lined by hypertrophied cells showing Hurthle-like alteration. The colloid is thin; reduced 40% from mag. × 600.

the periphery of the lobules and first manifested itself by hyaline thickening of the stromal reticular fibers and capillary walls. Lastly, the strangulated acini disappeared. In some situations there resulted diffuse scarring of the gland, and in other places there was progressive reduction in the size of the functional lobules, with apparent increase in the thickness of the interlobular septa. There never was evidence of active proliferation of fibrous stroma. In conjunction with these changes the lymphoid nodule formation and the lymphocytic infiltration receded.

It appeared that with the onset of spontaneous involution the glands initially increased in size and weight but in the late stage became small and sclerosed but did not adhere to surrounding structures.

Clinicopathologic Correlation

It is of interest to note that the average age of the early Group A was approximately 10 years less than the average age of the late Group D. In one case, we had the opportunity to study thyroid tissue removed nine years after previous subtotal thyroidectomy for struma lymphomatosa, and there was no marked change in the histologic pattern. The age differences of the various histologic groups suggest that spontaneous involution of the microfollicular gland may be a prolonged process of many years' duration.

Many patients were aware of the presence of goiter for variable periods and in several instances for as long as 30 years. Forty-three per cent had recognized glandular enlargement for five years or more. New complaints, either onset of toxicity, glandular enlargement, pressure effects, or hypothyroid features noted in the immediate preceeding few weeks or months precipitated medical aid in 41%.

The known presence of goiter in about half of these patients for at least five years, and in many instances recalled since childhood or adolescence, would indicate that a diffuse nontender symptomless goiter usually preceded the onset of spontaneous involution. What is the nature of this precursor? The logical antecedent struma, supported by study of the basic acinar pattern, is diffuse predominantly microfollicular goiter (parenchymatous or adolescent goiter).

Comment

What is the etiology of microfollicular goiter? What precipitates microfollicular hyperplasia? What facilitates or initiates the onset of spontaneous involution? None of these questions can be answered satisfactorily.

Microfollicular goiter has a definite geographic distribution. This type of struma occurs most frequently in high-altitude endemic goiter areas. In the United States, The microfollicular type of goiter is infrequent as compared with the macrofollicular variety. At Wichita, Kan., Hellwig[5] reported a 13.9% incidence of microfollicular goiters in surgical material. An 8.3% incidence in Wisconsin and a 6.8% incidence in Ann Arbor, Mich., of microfollicular goiter were recorded by Coller.[1] It may be that a relative or absolute lack of a specific element as yet unrecognized will prove responsible for development of microfollicular goiter. McCarrison[9] was able to produce lymphadenoid goiters in rats by a deficient diet.

Marine's classical experiments demonstrated the relation of iodine deficiency to macrofollicular goiter, but there has been an inadequate experimental attack on the problem of microfollicular goiter.

Identical nests of microacini have been previously described in diffuse exophthalmic goiter by Kocher, Rienhoff,[12] Hellwig,[5] Parmley and Hellwig,[11] and Goldberg and Davson.[3]

The available routine sections of 50 consecutive cases of toxic diffuse goiter then were reviewed. In 16 instances, small hyperplastic islands of microacini were found embedded in diffusely hyperplastic macrofollicular parenchyma. Surely, the percentage of cases with microfollicular foci in this study would be increased if multiple sections from multiple blocks of each thyroid gland

had been prepared. This brief survey again demonstrated the frequent occurrence of two variants of acinar hyperplasia in toxic diffuse goiter.

Rienhoff [12] believed the hyperplastic microfollicular foci seen in diffuse toxic goiter represented zones of hypoinvolution with temporarily decreased blood supply. Goldberg and Davson [3] concurred with this interpretation and enlarged it to include similar foci of microfollicular hyperplasia seen in adenomatous goiters. Parmley and Hellwig believed the microacini were the product of overstimulation by thyrotropic hormone, with resultant inability to store colloid. McClintock and Wright [10] stated that microacini of Hashimoto's disease were atrophic structures.

Each of the cases of diffuse microfollicular hyperplasia also showed zones of macrofollicles. If the patient had not received iodine therapy, the hyperplastic macrofollicular variant was identical with classical untreated exophthalmic goiter. The acini were large and irregular in outline and contained faintly eosinophilic colloid. The lining epithelial cells were tall and columnar, with rounded basal nuclei and abundant eosinophilic cytoplasm. Active epithelial mitosis, epithelial stratification, and papillary infolding were apparent. The cases that had received iodine therapy showed in the macrofollicular regions the well-established features of involution. The acini were distended, lined by flattened cells, and filled with deeply eosinophilic layered colloid. The hyperplastic microfollicles showed no response to iodine administration.

With struma lymphomatosa, some investigators vigorously deny and others faithfully record the preoperative presence of clinical hyperthyroidism. It would seem that the difference in clinical history is a reflection of the variation in proportion of hyperplastic microfollicular and macrofollicular elements. Curious admixtures of clinical hypo- and hyperthyroid activity also appeared. The atypical thyrotoxicosis that Hertzler described and the placid hyperthyroid patients that Crotti recorded are curious clinical images of a particular balance between hyperplastic micro- and macrofollicular elements. It is also necessary to emphasize that the clinical status of these patients, because of therapeutic or spontaneous glandular involution, will alter with time. In the end of involution all glands will be hypoactive.

In the process of case selection of struma lymphomatosa, there were 28 grossly nodular goiters with histologic features sufficiently diffuse and characteristic to have been misdiagnosed as struma lymphomatosa (Hashimoto). I excluded these cases from this series because they were grossly nodular, a feature most authorities will not accept in their concept of Hashimoto's disease. All of these nodular goiters were micromacrofollicular glands with marked lymphoid infiltration characteristic of early involution of the microfollicular hyperplasia, with focal or diffuse Hurthle-like epithelial-cell alteration.

In addition, a brief study of adenomatous colloid goiters revealed that they too were the products of variations in number and activity of these two types of thyroid follicles, the macrofollicle and the microfollicle. These glands represented the end result of involution of macromicrofollicular goiters. Goldberg and Davson and Lindsay et al. have recorded the presence of microfollicles in adenomatous and colloid goiters.

Thus, it is apparent that diffusely hyperplastic glands may show variation in the balance of two types of acinar structure. At one extreme, the gland will reveal pure diffuse macrofollicular hyperplasia; at the other extreme, the gland will present a pure diffuse microfollicular pattern. The intermediate stages of balance will form two major groups; macrofollicular hyperplasia is the dominant feature in one, and microfollicular hyperplasia is the prominent finding in the other. The distinction between these two groups will fade as the proportion of two acinar variants tends to equalize. Thus, diffusely hyperplastic thyroid glands

Interrelationship of Different Histologic Types of Goiter

Acinar Structure *	Type of Goiter
Pure Macro	
Macro+	Toxic diffuse goiter
Macro—	Diffuse colloid goiter
Macro micro	
Macro+micro	Toxic diffuse goiter with foci of lymphoid follicle formation
Macro+micro+	Toxic diffuse goiter with small fetal adenoma
Macro—micro—	Adenomatous colloid goiter with foci of { Lymphocytic infiltration / Chronic thyroiditis
Macro—micro+	Adenomatous colloid goiter with fetal adenoma
Micro macro	
Micro+macro—	Diffuse parenchymatous goiter with colloid adenoma
Micro+macro+	Parenchymatous goiter with foci of hyperplasia (toxic?)
Micro—macro—	Parenchymatous goiter with foci of { Lymphocytic infiltration / Chronic thyroiditis
Micro—macro+	Lymphadenoid goiter with foci of hyperplasia
Pure Micro	
Micro+	Adolescent or parenchymatous goiter
Micro—	Struma lymphomatosa

* + indicates hyperplasia; —, involution.

may be subdivided into four major groups and schematically represented as follows:

Diffuse hyperplasia
- (1) Macrofollicular
- (2) Macromicrofollicular
- (3) Micromacrofollicular
- (4) Microfollicular

In this scheme we have indicated that diffuse macrofollicular hyperplasia is identical with diffuse toxic goiter (Grave's disease). The macrofollicular variant is associated with thyroid hyperfunction and responds to iodine therapy, in contrast to the microfollicular variant, which is associated with glandular hypofunction and is not dependent on iodine. Clinically, as we proceed from pure macro- to microfollicular types, we can expect progressive reduction in thyroid function and in clinical response to iodine therapy. The interrelationship of the various types of goiter are schematically represented in the Table.

Furthermore, this concept of two functionally and morphologically distinct types of acini is well suited to the classification of both adenomata and carcinomata of the thyroid gland.

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Morphologic and Histochemical Changes in Postpartum Uterine Blood Vessels

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No recent reports of the morphologic features of postpartum vascular changes or articles utilizing modern histochemical procedures to demonstrate them were found in the literature. Gardner and Goodall,[1] as early as 1906, recognized changes in postpartum vessels and distinguished these changes from the vascular changes of arteriosclerosis. In 1910, a monograph by Goodall [2] related to involution of the puerperal uterus described the postpartum changes in uterine vessels as they were seen with the aid of Weigert's and Van Gieson's stains. Shaw,[3] in 1914, confirmed much of Goodall's work and, in addition, stated that if isolated vessels in the uteri of nulliparous women showed changes, they probably were due to early abortions. In 1919, Schwarz [4] studied 100 uteri which were enlarged and caused either pain, hemorrhage, or leukorrhea or a combination of these symptoms and confirmed the earlier findings of Goodall and Shaw.

Materials and Methods

Thirty-seven patients who died during the postpartum period after full-term pregnancy were used in this study. Four patients died during or immediately after delivery; five, within 24 hours; ten, in 1 to 7 days; eight, in 1 to 3 weeks, and the remaining ten, within 9 months after delivery.

Seven postpartum uteri after abortions also were examined. The length of gestation in these patients varied from four to seven months. Death occurred in two days to four and one-half months after the abortion.

The clinical records of all of the patients were reviewed to determine parity. For 26 of the 37 patients with full-term pregnancies, it was their only pregnancy. Multiparity of the remaining patients ranged from two to nine pregnancies. Four of the seven patients who had abortions had had only one pregnancy. Of the other three patients, two were parous 2 times and one was reported to be parous 15 times. All patients studied were less than 37 years of age; average age, 23.5 years.

Several routine blocks of tissue were selected from the uterine fundus at necropsy. In all cases, at least one block included the placental site. Blocks also were examined from the cervix, Fallopian tubes, and ovaries. The tissues were formalin-fixed, and the sections were stained with hematoxylin and eosin.

Three cases, all postpartum full-term first pregnancies, were specially selected for intensive histochemical investigation. One patient was 7 hours post partum; one, 15 days, and the third, 30 days. The following stains were used: Weigert's elastic stain, Verhoeff's elastic stain, periodic acid-Schiff stain with alcian blue counterstain, toluidine blue metachromatic stain, von Kóssa's stain, and Van Gieson's and Masson's trichrome stains for connective tissue.

Results

The large blood vessels supplying the placenta undergo the most marked changes. Adjacent vessels in the fundus will show these changes to a moderate degree, and the vessels in and around the cervix, Fallopian tubes, and ovaries usually show only slight changes in the postpartum period. The degree of change, therefore, appears to correspond to the extent to which the vessels have increased in size during pregnancy.

At, or shortly after, delivery the arteries and veins contract and collapse. In the veins the longitudinal muscle bundles segment so that columns of muscle are present in the wall and bulge into the lumen. In this way the lumen becomes irregular and,

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in some of the cul-de-sacs formed, endothelial surfaces are approximated. Collapse and contraction of the walls of the arteries also occur. However, the muscularis of the media does not show the marked segmentation present in veins. In the best examples found, the lumina of these vessels are stellate in cross section; this change is most strikingly seen in the uterus three to three and one-half hours after delivery.

The endothelium in the arteries swells and bulges into the lumen, forming a scalloped edge. Beneath many of the endothelial nuclei vacuoles form. At about 12 hours post partum, the approximated endothelial surfaces begin to fuse. The pattern of the fusion determines the position of the new lumen. In many cases the vessels have an eccentric lumen or, where fusion has occurred at the mouth of a cul-de-sac, a small lumen may persist in addition to the main channel. Where fusion does not occur, the vessels have lumina which appear stellate or slit-like in cross section. Such fusion as will occur has usually occurred by the sixth day.

In our material, thrombosis and canalization of the thrombus was not frequent and, when it did occur, was restricted to the large veins of the inner portion of the uterus, particularly adjacent to the area of placental implantation. Some of the large arteries supplying the placenta showed complete obliteration and remained as hyaline scars marking the placental site.

The elastic tissue of the internal elastica and adventitia show marked changes. At, or shortly after, delivery when the vessels contract, the elastic fibers swell to three to four times normal size, become markedly eosinophilic with use of routine hematoxylin and eosin stain, and lose the normal refractile quality. In routine stains of uterine arteries shortly after delivery, the internal elastica appears increased in width beneath the endothelium and the adventitia is seen as a broad homogeneous zone which surrounds the vessel and blends with the interstitial tissue of the myometrium. With Weigert's and Verhoeff's elastic tissue stains, the elastic tissue fibers in these locations stain moderately red to deep brick red instead of the normal black. Within seven hours post partum, these changes are marked and persist beyond the 15th postpartum day, while by the 30th postpartum day the normal staining qualities have been regained. Between the 15th and 30th days post partum a marked reduction in the elastic tissue takes place; however, excess elastic tissue usually remains, especially around the adventitia and occasionally

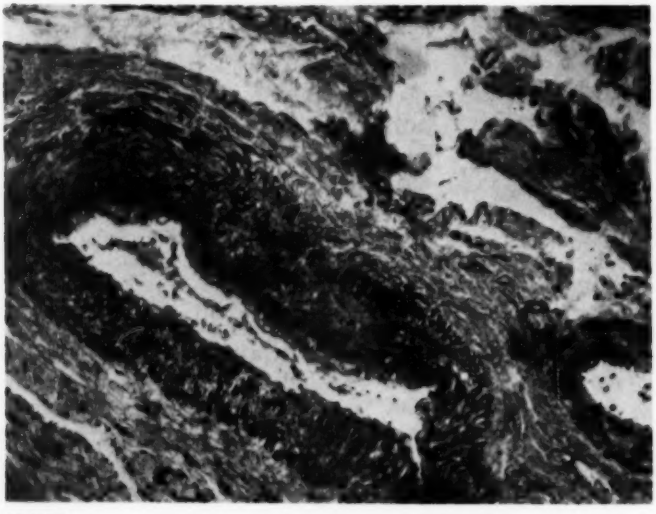

Fig. 1.—Uterine artery six hours after delivery. There is loss of polarity of the smooth-muscle cells, with thickening and hyalinization of the internal elastic lamina. Hematoxylin and eosin; reduced 15% from mag. × 78.

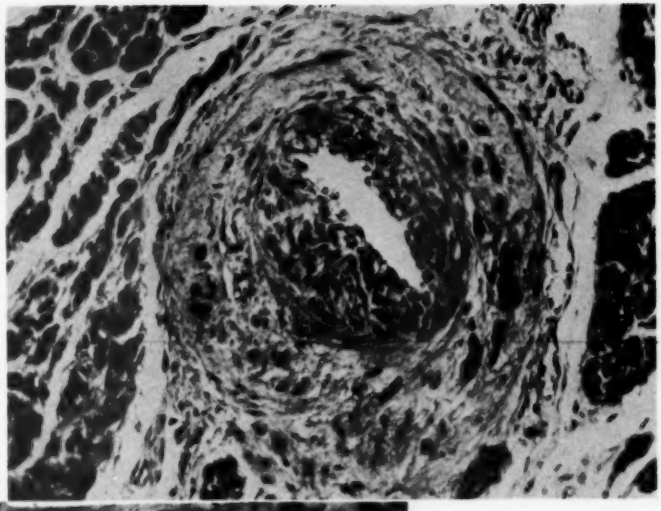

Fig. 2.—A small uterine artery five days post partum. Degenerating smooth muscle can be seen to some extent around the circumference of the vessel; however, it is much more marked on the left side of the lumen. The smooth muscle adjacent to the lumen shows loss of polarity. Adventitial connective tissue is loose and abundant. Hematoxylin and eosin; reduced 15% from mag. × 205.

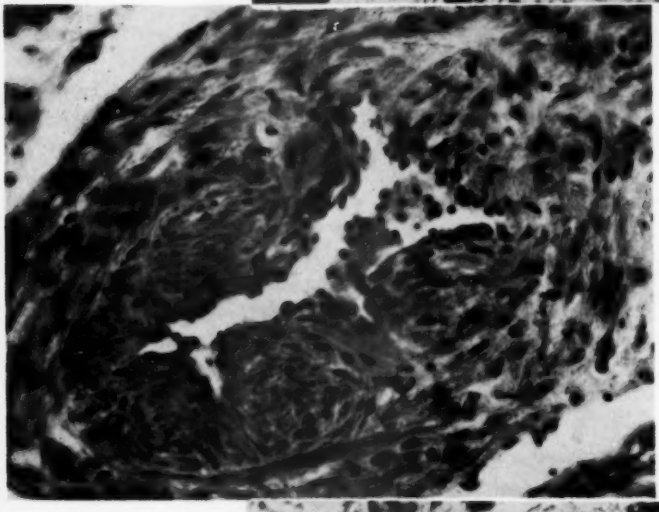

Fig. 3.—A small uterine artery 15 days post partum. The muscularis is beginning to regain some normal circumferential orientation. The adventitial connective tissue is less abundant than in Figure 2. Hematoxylin and eosin; reduced 15% from mag. × 295.

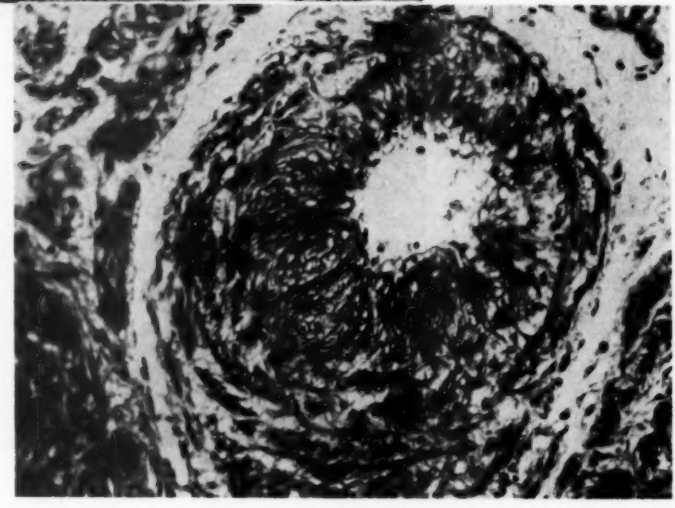

Fig. 4.—Small arteriole seven hours post partum. Marked contraction has occurred, approximating some of the endothelial surfaces. Intracellular edema of the endothelium. Hematoxylin and eosin; reduced 15% from mag. × 210.

Fig. 5.—A large uterine artery, 30 days post partum. The old elastic tissue persists in the adventitia, and the old internal elastic lamina is now separated from the new lumen by a broad band of subintimal fibrosis. Weigert's elastic tissue stain; reduced 15% from mag. × 80.

around the internal elastica, serving to differentiate the parous from the nulliparous uterus. At times, the entire old internal elastic lamina of arteries persists and encloses one or more new-formed vessels which have new internal elastic laminae.

Masson and Van Gieson stains accentuate the degree of change which occurs in the collagenous tissue of the vessel wall. As in the elastic tissue, in a few hours after delivery some homogenization of collagen is observed. Fifteen days after delivery this change is marked but the collagenous tissue, unlike the elastic tissue, stains intensely, i. e., with Van Gieson's stain, deep red, and with Masson's stain, brilliant green.

The apparent increase in collagenous tissue is chiefly in the adventitia, although some increase may be found in all portions of the wall, especially in those arteries with marked reduction in the lumina. In the latter, large plaques of fibrous tissue may be found beneath the intima and bulging into the lumen. Single muscle fibers in these areas may appear to be surrounded by collagenous tissue. Thirty days after

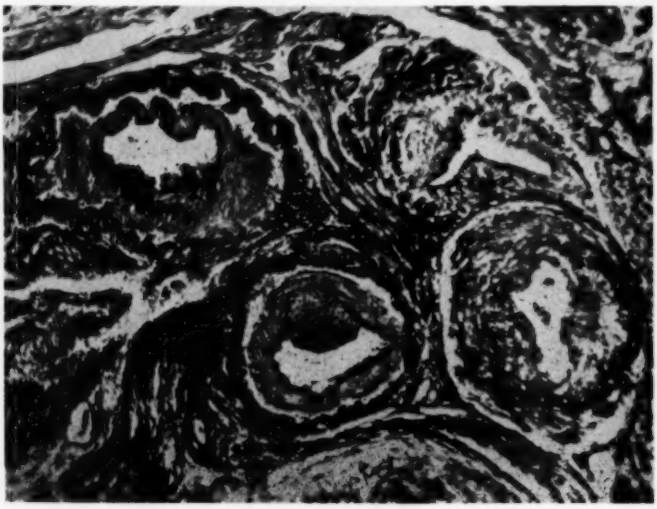

Fig. 6.—Thirty days post partum. Large amount of elastic tissue persists around the vessels. The small artery in the center shows splitting of the internal elastic lamina, and all vessels have irregular lumina. Verhoeff's elastic tissue stain; reduced 15% from mag. × 158.

delivery, the adventitial collagen is markedly reduced. In uteri showing poor involution, more elastic than collagenous tissue appears to remain in this area.

The periodic acid-Schiff stain showed staining reactions which paralleled the change in the collagenous tissue. The specimens obtained seven hours post partum showed diffuseness and homogenization of the polysaccharide-positive material within the walls of the vessels; 15 days post partum, the Schiff-positive material was more discrete, stained more intensely, and was largely confined to the subintimal and adventitial areas. Thirty days after delivery, a marked reduction in the Schiff-positive material in the adventitia was noted, while the subintimal zone remained unchanged.

Toluidine blue metachromasia was absent in the walls of the arteries seven hours post partum; it was only slight at 15 days but abundant 30 days after delivery. It was especially marked in vessels which were either largely or completely obliterated. In these vessels the metachromasia was especially marked in the subintimal areas. Von Kóssa's stain was negative in all sections examined.

Postpartum uteri from patients who had had premature delivery showed the changes, as did those from full-term pregnancies, providing gestation was at least six months in duration. If the pregnancy was terminated before six months' gestation, the changes were less marked. In many cases, involution appeared to be less complete in uteri after abortion than after full-term pregnancy. Possibly, the inflammatory changes usually present in these uteri adversely influence the normal involution. Although the group of uteri from patients with abortions was small, the conclusions probably are significant.

In the uteri of multiparous patients, the arterial changes were generally more marked, probably because of persistence of elastic tissue around the vessels after previous pregnancies.

Comment

The vessels in the parous uterus which are markedly enlarged at the time of delivery undergo rapid reconstruction consistent with the new functional demands of the uterus. The vascular component of uterine involution usually is complete within 40 to 44 days post partum.

These vascular changes are distinct from those of the senile or disuse type of arteriosclerosis found in postmenopausal uteri. In postmenopausal arteriosclerosis the entire circumference of the vessel is affected uniformly and the smooth muscle of the media is atrophic, while in postpartum arteriosclerosis the musculature is not atrophic but malformed in the regenerative process and the lumen is often eccentric. Postmenopausal parous uteri may exhibit both types of changes.

In the uteri with residual connective tissue around vessels, most of the tissue is elastic in type. The reason for this is not readily apparent; however, the process, probably enzymatic, that removes excess tissue might be expected to be less effective on elastic tissue than on collagen.

The metachromasia with toluidine blue is of interest, since this change has been noted in arteriosclerotic vessels in association with degeneration of elastic fibers.[5,6] However, in the example of postpartum arteriosclerosis the metachromasia was not marked in the early stages of alteration of the elastica but was most prominent when the elastic tissue had regained the normal staining quality after use of Weigert's and Verhoeff's stains.

Alterations in the mucopolysaccharide ground substances, as confirmed by the periodic acid-Schiff and toluidine blue stains, merit special attention, since they have been associated with the degenerative changes of atherosclerosis and age in other arteries.[5-7] These changes were not attributed to the direct result of excessive functional demands, as all of the changes developed during the involutional period.

Summary and Conclusions

The appearance of postpartum uterine blood vessels is described as seen with hemotoxylin and eosin and other stains.

Shortly after delivery, marked contraction and collapse of the uterine vessels occur. The elastic tissue loses its normal staining properties for about 25 to 30 days. During this period, much of the excess elastic tissue is removed. After the 30th day the usual staining reaction is restored.

Abnormalities persist in postpartum vessels in the pattern and amount of elastic tissue and smooth muscle, and to less extent, of collagenous tissue.

Alterations in the mucopolysaccharide ground substance resemble those found in the degenerative changes associated with atherosclerosis and aging, but, unlike the latter, they do not occur during the time when the elastic tissue is most altered.

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A Case of Encephalitozoon-like Body Infection in Man

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Encephalitozoon was first described by Wright and Craighead (1922) from the brain and kidney of a rabbit having a motor paralysis. Levaditi et al. (1923) named this parasite Encephalitozoon cuniculi, and Cowdry (1929) found the same parasite in mice. Perrin (1943) reported the spontaneous infection with this parasite in guinea pigs, rats, and mice. In the course of our studies on Toxoplasma, which were begun in 1952, Tazaki (1956) found Toxoplasma-like bodies in the cells of the peritoneal fluid of mice which had been given injections intraperitoneally with brain and liver emulsion from Japanese sparrows. Further studies revealed these Toxoplasma-like bodies to be Encephalitozoon which were morphologically and immunologically distinct from Toxoplasma (Tazaki, 1957).

Subsequently, on Aug. 26, 1957, a patient was brought to this clinic, and Encephalitozoon-like bodies were found in the spinal fluid and urine. The clinical and parasitological aspects of this infection constitute the essence of this paper.

I. Clinical Observations

The patient was a 9-year-old boy, a resident of Atsugi, Kanagawa Ken, Japan. He presented symptoms of recurrent fever, loss of conciousness, and headache. No previous history seemed contributory. The tuberculin reaction was negative, and he had received BCG vaccine four months before being brought to this clinic.

Family history indicated his father was a farmer keeping cows, pigs, and chickens, all presumably healthy. No dogs or cats were kept, although the patient kept pigeons which he frequently fondled.

The first attack of the present illness occurred on Aug. 26, 1957. At 2 a. m., after urination, he vomited and had a spasmic convulsion. The physician who was called indicated later that the body was rigid, the head and eyes were turned to the left, and the patient was unresponsive. The spasms terminated only to recur. The patient was admitted to the hospital two hours after the initial attack. On admission, the patient was somnolent and only slightly responsive. The spasms had terminated. Temperature was 38 C (100.4 F); respirations and pulse, normal; blood pressure, 108/60. There was no paralysis of the ocular muscles or nystagmus, and reflex of pupil was normal. Eyes were, however, pulled to the right for one or two minutes. The tendon reflex was weak in general, and no pathological reflexes or signs (Babinsky, Trousseau, Oppenheim) were seen. No meningeal symptoms (Kernig, head retraction), paralysis, or spasms in arms and legs were evidenced. Two hours after admission, the patient gradually recovered consciousness but complained of severe headache and vomited occasionally.

The findings on clinical laboratory examination at the time of admission and later on are shown in Figure 1 and Tables 1 and 2. The cerebrospinal fluid pressure was 210 mm., and on withdrawal of 15 ml. of the fluid it dropped to 140 mm. The cell count was 116/3. Yokota's reaction, which is effective for the diagnosis of Japanese encephalitis, was normal. The sediment of the fluid was not examined. On blood examination a mild leukocytosis and anemia were found. Sedimentation rate was 118 mm. in 1 hour and 127 mm. in 2 hours. Tuberculin reaction was 0/7×8. Bacterial cultivation from arterial and venous blood was negative. Urinalysis was negative for protein, bilirubin, and urobilin. Wassermann reaction was negative.

From the clinical findings stated above, epilepsy and Japanese encephalitis were initially suspected. Fever, leukocytosis, increased sedimentation rate, and absence of subsequent convulsions suggested some infectous disease, and 800,000 units of penicillin was injected daily for five days, beginning on the day after admission. The patient suffered from headache and fever and sometimes vomited. He looked apathetic but had no symptoms attributable to meningeal stimulation. None of the pathological conditions of eye, such as papilledema,

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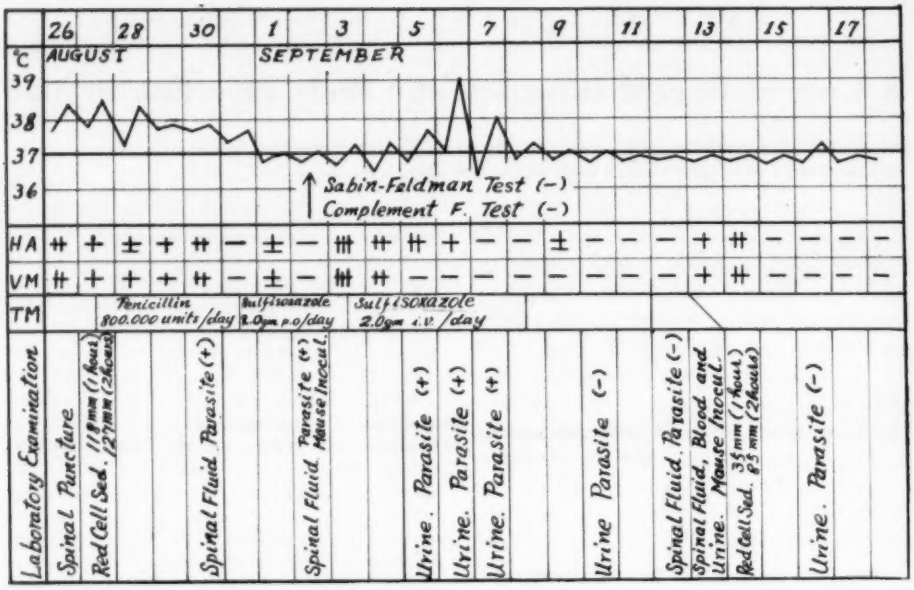

Fig. 1.—Course of the clinical symptoms of the patient harboring the Encephalitozoon-like bodies. *HA* indicates headache; *VM*, vomiting; *TM*, treatment, and parasite (+) or (−) in smears.

bleeding, or retinal exudate, was observed. The field of vision was also normal.

On Aug. 30, the second spinal puncture was made. The cell count increased to 336/3, and the Nonne-Apelt reaction became negative. On examination of the sediment stained with Ziehl-Neelsen reagent, small oval bodies were found free in the fluid. Each had a red nucleus and blue cytoplasm and looked somewhat like Toxoplasma. With Giemsa stain each body had homogenous blue cytoplasm and a red or purple nucleus. Their size was 2.7μ-3.0μ by 1.2μ-1.8μ (Fig. 2*A*). As these bodies were considered to be Toxoplasma, penicillin injection was suspended and sulfisoxazole was given, 2.0 gm. daily. At that time the temperature was dropping and the patient's appetite was improving. On Sept. 2, the Sabin-Feldman dye test and the complement-fixation test for toxoplasmosis were done, giving negative reactions. On the same day, 20 ml. of cerebrospinal fluid was withdrawn, and after centrifugation for 20 minutes at 3,000 rpm, the sediment was injected into the peritoneal cavities of nine mice. The examination later revealed apparently the same bodies in the peritoneal fluid of these mice. A detailed description of this

TABLE 1.—*Clinical and Laboratory Observation on the Cerebrospinal Fluid from the Patient Harboring the Encephalitozoon-like Bodies*

		Aug. 26	Aug. 30	Sept. 2	Sept. 12
Pressure, mm.	First	210	170	180	260
	Final	140	80	30	160
Amount withdrawn, ml.		15	12	20	10
Cells, No.		116/3	336/3		83/3
Nonne-Apelt		(−)	(+)		(−)
Pandy		(+)	(+)		(+)
Protein, mg/dl.		20			
Glucose, mg/dl.		100			
Yokota's reaction		Normal			
Oval bodies (direct smear)			(+)	(+)	(−)

TABLE 2.—*Clinical Studies on Blood from the Patient Harboring Encephalitozoon-like Bodies*

	Aug. 26	Sept. 6
Hemoglobin (Sahli)	60%	
Red cells	301×10⁴	
White cells	11,000	7,600
White Cell Differential Count		
Neutrophil		
Stab cells	3.5%	
Segmented cells		
II	27.5%	21.5%
III	22.0%	33.5%
IV	16.0%	20.0%
V	4.0%	1.5%
Eosinophil	1.0%	0.5%
Monocyte	2.5%	2.0%
Lymphocytes	23.5%	21.0%

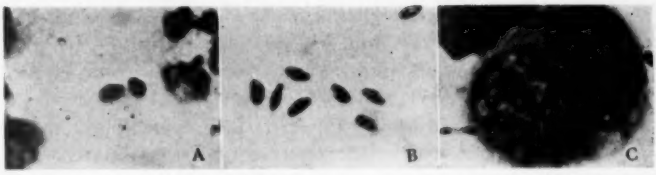

Fig. 2.—Encephalitozoon-like bodies from the patient and animals given experimental inoculations. *A*, the oval bodies in the cerebrospinal fluid of the patient. *B*, the oval bodies in the peritoneal fluid of a first-passage mouse. *C*, the colony of the oval bodies in a macrophage cell in the peritoneal fluid of a mouse of the third passage.

aspect is made below. X-ray examination of the brain on Sept. 3 did not show any calcification.

After the spinal puncture on Sept. 2, headache and vomiting became rather intense and frequent and the patient could eat and drink only a little. Sulfa-isoxazole was given intravenously. On Sept. 5, urinalysis revealed the same bodies which had been recovered from the cerebrospinal fluid. They were found for three successive days in the urine, but bladder symptoms, such as tachyuria and pain during micturation, were not evidenced. Urine was negative for protein or showed only a trace. Cylinders were not detected, and a small number of leukocytes and pavement epithelial cells were seen. The kidney was never palpable.

On Sept. 6, the patient had a sudden fever, with temperature of 39 C (102.2 F). This was due to a recrudescence of otitis media which he had three years ago, and by local treatment the temperature dropped to normal. The oval bodies which were found in the cerebrospinal fluid and urine were not seen in the secretion from the ear. A thick blood smear made on Sept. 8 did not show any oval bodies. On Sept. 12, the oval bodies were no longer seen in the smears of cerebrospinal fluid and urine, but the injection of these fluids and also of blood into the peritoneal cavities of mice proved the presence of the bodies in those materials. The findings in these animal inoculations will be stated in detail below.

After Sept. 12, the patient became gradually more active and had no complaint except fever and vomiting on two occassions. Subsequent examinations of urine revealed no oval bodies, and the red cell sedimentation rate returned to 35 mm. in one hour. The patient was discharged on Sept. 19, or 24 days after the onset of his symptoms. Though he complained of headache for several days after discharge, no symptoms appeared later. The Sabin-Feldman dye test and complement-fixation test for Toxoplasma made on Sept. 20 were negative.

II. Animal Inoculations

As stated above, materials freshly obtained from the patient were inoculated into mice on two different occasions. In the initial inoculation, which was made on Sept. 2, twenty milliliters of the cerebrospinal fluid of the patient was centrifuged at 3,000 rpm for 20 minutes and the sediment was inoculated intraperitoneally into nine mice, each mouse receiving a 1.0 ml. suspension of the sediment (Fig. 3). All animals showed abdominal distention due to ascites two to three weeks after the inoculation, and the oval bodies were found in the peritoneal fluid of four mice killed on Sept. 15, Sept. 24, and Oct. 4. These bodies were generally extracellular (Fig. 2*B*). The peritoneal fluid of each of these four (positive) mice was transferred to two new mice, all of which showed many oval bodies in the macrophage cells of their peritoneal fluid three to four weeks after the transfer (Fig. 2*C*). The remaining five (negative) mice were killed on Oct. 29, and their brains, livers, and spleens were emulsified and mixed together (referred to

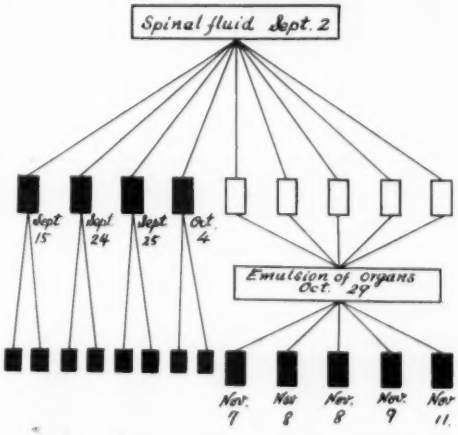

Fig. 3.—Inoculation of the cerebrospinal fluid of the patient into mice, and subsequent transfers. Black squares indicate positive mice, and white squares indicate negative mice.

as three-organ emulsion hereafter) and inoculated intraperitoneally into five mice in the amount of 0.5 ml. each. These mice were killed 9, 10, 10, 11, and 13 days later. All mice were found to be infected, the oval bodies being found in macrophages of the peritoneal fluid.

In the subsequent inoculation which was made on Sept. 12, cerebrospinal fluid, blood, and urine of the patient were used as inoculation materials. None of these materials showed the oval bodies by direct smear examinations as stated above. The procedures and results of these inoculations were as follows.

Cerebrospinal Fluid.—About 20 ml. of the fluid was centrifuged at 3,000 rpm for 20 minutes, and the sediment suspended in about 5 ml. of the same fluid was injected into five mice in the amount of 0.5 ml. each. In three of the mice, the oval bodies were found in their peritoneal fluid 27, 27, and 30 days after the inoculation, respectively (Fig. 4). In the remaining two mice, the oval bodies were not found until Oct. 28 (46 days after the inoculation), when they were killed and their three-organ emulsion was injected into five mice. One of the mice already showed the oval bodies in the cells of peritoneal fluid on Nov. 11 (13 days after inoculation), and all of the other four mice were found to be infected three days later.

Blood.—Sixteen milliliters of blood was withdrawn from the patient, and four milliliters of 5% sodium citrate solution was added to it. This mixture was injected intraperitoneally into five mice, each receiving 1 ml. of the mixture. Two of them were found to be infected 30 and 33 days after the inoculation, respectively (Fig. 5). The remaining three in which the oval bodies were not found were killed 46 days after the inoculation, and their three-organ

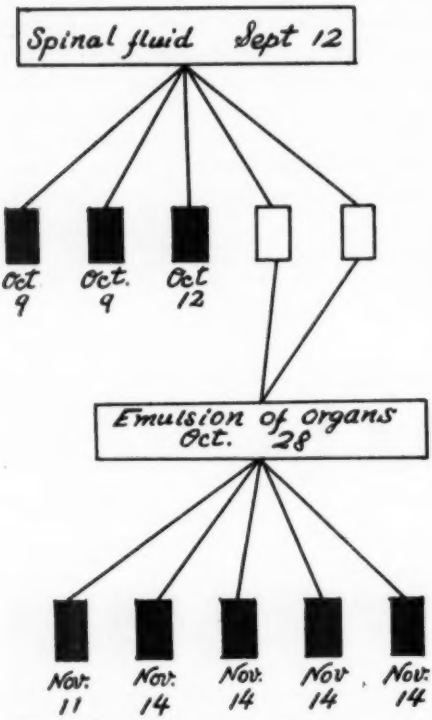

Fig. 4.—Inoculation of the cerebrospinal fluid of the patient into mice, and subsequent passages.

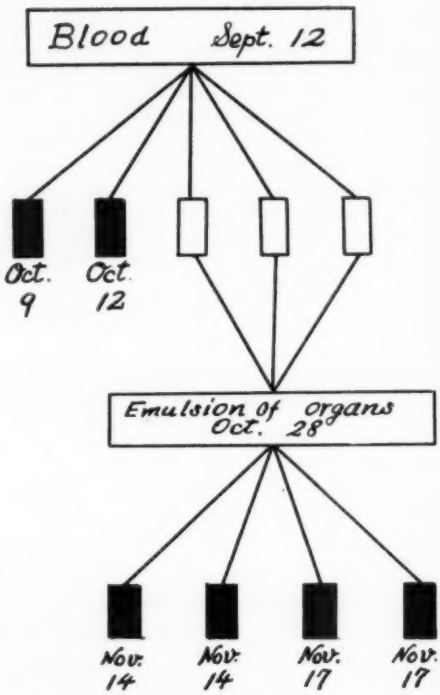

Fig. 5.—Inoculation of the blood of the patient into mice, and subsequent passages.

emulsion was injected intraperitoneally into four mice. Two of them showed the oval bodies in the cells of peritoneal fluid 17 days after the inoculation, and the other mice were found infected 3 days later.

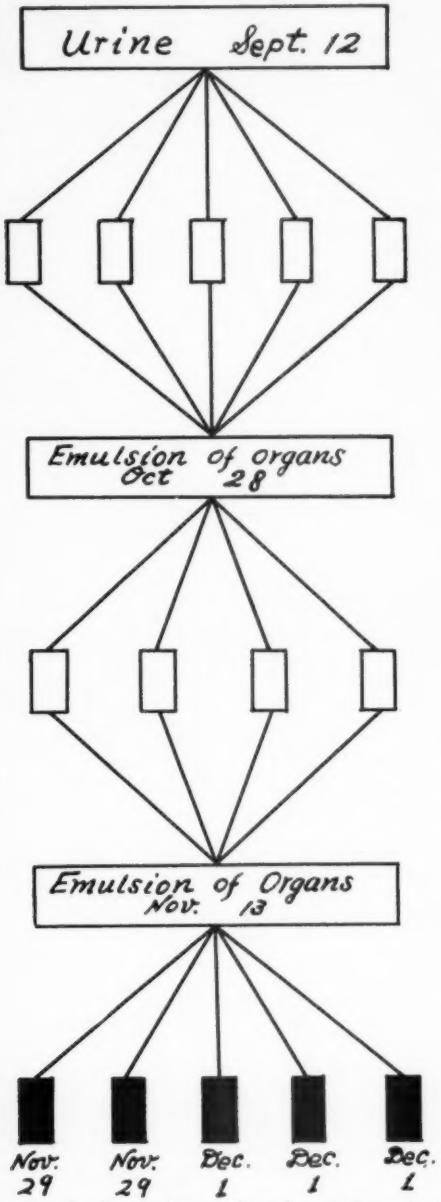

Fig. 6.—Inoculation of the urine of the patient into mice, and subsequent passages.

Urine.—Urine was centrifuged, and the sediment was repeatedly washed with isotonic saline by centrifugation. The final sediment was suspended in saline, and 1.0 ml. of the suspension was injected intraperitoneally into each of five mice. No oval bodies were found in any of these five mice until Oct. 28 (46 days after the inoculation), when they were killed and the three-organ emulsion was injected into four mice (Fig. 6). As these four mice of the second passage did not show any oval bodies in their peritoneal fluid until Nov. 13 (16 days after the inoculation), they were all killed on that date, and the mixed emulsion of their three organs were injected into five mice. The oval bodies were found in two of these mice 16 days after the inoculation and in the remaining three mice 2 days later.

To assure against the possibility of spontaneous infection of the mice, 30 controls were used. Livers, brains, and spleens were emulsified and injected into 27 mice. All remained negative through that and the second passages.

III. Morphology of the Oval Bodies in Mice

The morphology of the oval bodies found in the cerebrospinal fluid and urine of the patient was mentioned above. Those bodies found in the peritoneal fluid of mice given inoculations showed little difference in morphology from those found in man. In the peritoneal fluid of the first-passage mice, the bodies were extracellular and oval in shape, and they measured 2.3μ-3.4μ in length and 1.8μ-2.8μ in breadth. The protoplasm was homogeneous and stained blue by Giemsa stain; the nucleus was spherical or oval in shape, stained red or deep purple, and situated eccentrically (Fig. 2*B*). In the second and subsequent passages in mice, most of the oval bodies were found in macrophages of the peritoneal fluid, a few being found free in the fluid. The number of the bodies in one host cell was variable, being several in some cases but mostly too

numerous to be counted. The individual organism was smaller than the extracellular ones, measuring 1.5μ-3.0μ in length and 1.4μ-2.0μ in breadth. The shape and internal structure were the same in the free forms (Fig. 2*C*). By Gram stain, the oval bodies showed definite positive reaction, while the Toxoplasma (RH strain), stained as a control on the same slide, showed negative reaction.

IV. Pathogenicity of Mice

As briefly stated above, mice given inoculations of the oval bodies showed gradual distention of the abdomen one week after the inoculation. The abdominal distention was due to ascites. The oval bodies first appeared at that time and increased gradually in number and attained maximum at about two weeks after inoculation. They decreased in number later and disappeared four to five weeks after the inoculation. The amount of the peritoneal fluid was maximum (0.5-3.0 ml.) at the fourth or fifth week. It was sticky at first and became more fluid later when the oval bodies disappeared. At the fifth or sixth week the amount of fluid diminished definitely. Some of the infected mice became inactive, showed ragged hair, and had anorexia and diarrhea at the third or fourth week. None died of the infection except several young mice which were given injections of a large amount (1.0-1.5 ml.) of the infected peritoneal fluid. In the infected mice, liver and spleen became three times as large as normal. Enlarged spleen and liver were brittle and purple-red in color. They became smaller and were of normal size four to five weeks after the inoculation. Virulence of the oval bodies did not increase by serial passages.

V. The Sabin-Feldman Dye Test of the Infected Animals

The peritoneal fluid of 10 mice which had been given inoculations of the oval bodies two to three weeks previously was collected and suspended in isotonic saline (2 ml. of saline per mouse), and 2 ml. of this suspension was inoculated into each of 10 guinea pigs. Blood was withdrawn from these guinea pigs two to four weeks after the inoculation, and the Sabin-Feldman dye test was carried out. All guinea pigs gave negative tests. That the infection of oval bodies in these guinea pigs was successful was confirmed later by the inoculation of the emulsion of their liver, spleen, lung, and kidney into mice. As controls, guinea pigs were infected with Toxoplasma (RH strain) and their sera were tested. They all showed positive Sabin-Feldman test, the titer varying from 1:16 to 1:1,024.

Comment

Oval uninucleate organisms with homogeneous protoplasm were found in the cerebrospinal fluid and urine of a patient who had attacks of convulsions and fever. They were also recovered from peritoneal fluid of mice given experimental inoculations of cerebrospinal fluid, blood, and urine of the patient. This strain has been maintained by serial passages through mice. The organisms are similar to Toxoplasma and Encephalitozoon in their morphology, localization, and multiplication in the host. The following comparative considerations of characteristics of the oval bodies under consideration suggest, however, that they are to be identified as Encephalitozoon rather than Toxoplasma:

1. Their size varies from 2.4μ-3.4μ in length and 1.8μ-2.8μ in breadth in the free (extracellular) stage and 1.5μ-3.0μ in length and 1.4μ-2.0μ in breadth in the intracellular stage. These sizes are far smaller than those of Toxoplasma and coincide with the size reported for Encephalitozoon.

2. Cytoplasm is homogeneous by Giemsa stain, as is characteristic of Encephalitozoon. Toxoplasma has a granulated cytoplasm.

3. They are Gram-positive. Encephalitozoon is also Gram-positive while Toxoplasma is Gram-negative.

4. They do not kill mice by experimental inoculation, and the virulence does not increase by repeated serial passages. This is also one of the characteristics of Encephalitozoon. Toxoplasma is usually highly pathogenic to mice. Even if their infection may not be fatal in the first several passages after

isolation from spontaneously infected animals, their virulence gradually becomes higher by serial passages.

5. Sabin-Feldman dye tests were all negative both in the patient and in the animals experimentally infected. The patient serum was also negative to the complement-fixation test for Toxoplasma.

All of the features cited above indicate that the organisms under consideration should belong to Encephalitozoon rather than to Toxoplasma. We have been maintaining several strains of Encephalitozoon isolated from sparrows and wild rats, and Kyo (1957) in our laboratory carried out cytochemical studies on these strains and described morphological differences between Encephalitozoon and Toxoplasma. The organisms under consideration coincide in all respects with his description of Encephalitozoon and not with Toxoplasma. According to Wright and Craighead (1922), Goodpasture (1924) and Levaditi et al. (1923), Encephalitozoon is discharged into the urine of the infected rabbits and the infection is transmitted by the injection of the urine into clean rabbits. In the present case, the organisms were found in the urine of the patient and mice were successfully infected by the injection of the urine. Those features also indicate that the organisms coincide with Encephalitozoon.

Summary

Encephalitozoon-like organisms were found in the cerebrospinal fluid and urine of a nine-year-old boy who had cerebral symptoms. The organisms were oval in shape and 2.7μ-3.0μ in length and 1.2μ-1.8μ in breadth. They had homogeneous light blue cytoplasm with deep purple or red nuclei on Giemsa staining.

On injection of the cerebrospinal fluid, blood, and urine of the patient into mice, the same organisms were found in the peritoneal fluid of the animals. This strain of organisms could be maintained by serial passages through mice. In the first passage into mice of the material of the patient, the organisms were found to be extracellular in the peritoneal fluid and their size was 2.3μ-3.4μ in length and 1.8μ-2.8μ in breadth. After the second passage, they were found mostly as intracellular colonies and the size of the individual parasite was a little smaller than the extracellular organisms, measuring 1.5μ-3.0μ in length and 1.4μ-2.0μ in breadth. They were Gram-positive. Serum of the patient, as well as that of the experimentally infected animals, never gave a positive Sabin-Feldman dye test.

These observations suggest the possibility that the organisms recovered from the patient are Encephalitozoon.

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Cytochemistry of Myelofibrosis with Myeloid Metaplasia in Relation to Spleen Changes

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Myelofibrosis with myeloid metaplasia (also known under many other names) is a condition characterized pathologically by gradual fibrosis and sclerosis of the bone marrow, with simultaneous proliferation of bone marrow elements in various organs, especially in the spleen and liver. It often occurs late in the course of polycythemia vera. In some patients, there is a history of prolonged exposure to myelotoxic agents, usually aromatic solvents. Not infrequently no etiological factors can be determined. Histochemical studies of the blood cells indicate that there is usually an increase of alkaline phosphatase of the mature neutrophils; however, a fair percentage of cases shows a low alkaline phosphatase content of these cells.[1,2] In our series of 23 cases, 7 (30%) had low values, with the rest showing greatly elevated levels.[2] Whether the case was postpolycythemic after chemical exposure or completely agnogenic did not seem to have any determining influence in this respect. The only difference observed between the two types of myeloid metaplasia was possibly the greater immaturity of the circulating white blood cells in the type associated with low alkaline phosphatase.

The clinical and pathological diagnoses of myeloid metaplasia do not always go hand in hand. Often sharp differences exist, and many cases labeled clinically and hematologically as myeloid metaplasia are diagnosed at autopsy as chronic granulocytic leukemia. This may in part be owing to a difference in the stage of the disease, which may show different pictures at different times. Similar diagnostic difficulties exist in relation to spleens removed surgically. This diagnostic divergence is somewhat perplexing and may indicate the necessity of reviewing or relaxing the criteria for the differential diagnosis between myeloid metaplasia and chronic granulocytic leukemia, especially in the case of the spleen. Some observers believe that strict diagnostic criteria should be employed in making the pathological diagnosis. For example, the persistence of lymphoid follicles in the spleen and the focal distribution of myeloid changes would indicate myeloid metaplasia, while the diffuse myeloid proliferation with obliteration of the lymph follicles would indicate chronic granulocytic leukemia.[3,12] Others[4-6] believe that myeloid metaplasia and chronic granulocytic leukemia cannot be separated by such rigid criteria and that a wide "borderland" exists between these two conditions into which they both imperceptibly merge.

The purpose of this study was to examine the surgically removed spleens of patients having the characteristic features of myelo-

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fibrosis with myeloid metaplasia, those with both high and low alkaline phosphatase content of neutrophils, and to see whether any histologic, cytologic, or cytochemic differences could be determined.

Material and Methods

The surgically removed spleens of four patients with myeloid metaplasia were the object of the study. The patients were diagnosed as having myelofibrosis with myeloid metaplasia by the following criteria:

1. Splenomegaly, splenic aspiration showing myeloid metaplasia.
2. Anemia, with nucleated red blood cells and immature white blood cells in the peripheral blood.
3. Repeated dry bone marrow taps and fibrosis of the bone marrow as shown by surgical biopsy.

Alkaline phosphatase of neutrophils (in peripheral blood) was estimated by use of the cytochemical method (azo dye) and scored according to Kaplow.[7] In our previous study[2] the normal range was 16-53. Two of the patients in the present study had elevated scores—one markedly so (257) and the other moderately (86). They constitute what we called cytochemical Type I of myeloid metaplasia. The remaining two patients had low scores (18, 9, and 7 on repeated examinations of one patient and 16, the other) and were called Type II myeloid metaplasia.

The spleens were weighed and examined within one to three hours of their removal; imprints were made, and sections were taken. Tissues were fixed in formol-saline, Zenker's solution, and acetone, and the following stains performed.

Imprints.—Alkaline phosphatase (Gomori-Takamatsu[8,9] and azo dye[7]), acid phosphatase (Gomori[10]), periodic acid-Schiff (P. A. S.) stain (with and without diastase digestion).

Sections.—Hematoxylin and eosin, phloxine-methylene blue, silver impregnation for reticulum (Gordon and Sweet), Mallory's stain for fibrous tissue, P. A. S. (1% malt diastase in 0.2 M phosphate buffer at pH 6.7 for three hours at 37 C), alkaline phosphatase (Gomori-Takamatsu), acid phosphatase (Gomori).

Four normal spleens removed for technical reasons during abdominal operations were examined in the same way, and they served as controls.

Results (Table)

Myeloid Metaplasia, Type I

Case 1 (alkaline phosphatase score 257). The spleen weighed 1,500 gm. Splenic imprint (Fig. 1) showed a marked increase of the cells of the granulocytic series, some of which were immature (blast cells 2.4%, myelocytes 3.2%). The normoblasts were very numerous (20%), and occasional

Differential Counts of Splenic Imprints

		Myeloid Metaplasia			
		Type 1		Type 2	
Alk. Phos. Score	Normal Value (16–53), %	(257)	(86)	(18)	(16)
Lymphocytes	75–90	50.8	48.0	24.0	20.0
Neutrophilic P. M. N.	5–15	16.0	5.2	8.0	10.4
Basophils	0–1	0.8	0.8	2.0	0.8
Eosinophils	0–1	0.4	0.8	0.4	0.4
Metamyelocytes	0–2	2.4	2.0	6.8	19.6
Myelocytes	0	3.2	3.2	7.6	40.0
Blast cells	0	2.4	1.2	6.0	6.4
Total of granulocytic series		25.2	13.2	30.8	77.6
Reticulum & "pulp" cells	0–1	4.0	1.6	10.0	0.0
Normoblast "A"	0	1.2	0.0	2.0	0.8
Normoblast "B"	0	2.8	4.4	10.0	0.4
Normoblast "C"	0	16.0	32.8	23.2	1.2
Total of normoblast series		20.0	37.2	35.2	2.4
Plasma cells	0–1	0	0	0	0
Total		100.0	100.0	100.0	100.0
Megakaryocytes	0	+	+	+—	+
Alk. phosph. of neutrophils	0–+	2+–4+	2+	0–1+	0–+

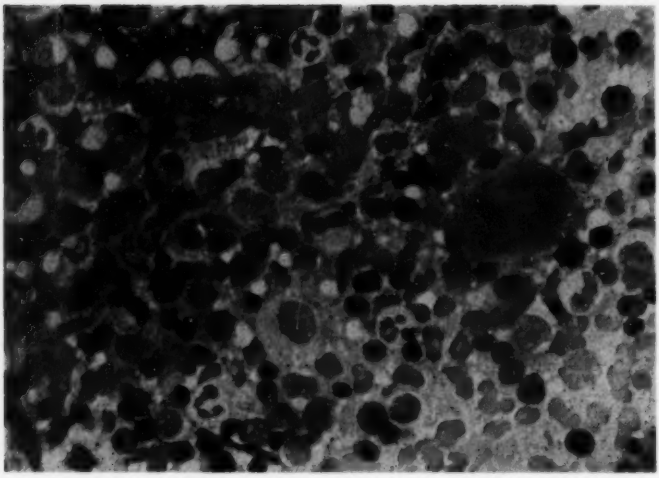

Fig. 1 (Case 1).—Splenic imprint. Lymphocytes are present in good numbers. In addition, myelocytes, metamyelocytes, mature granulocytes, nucleated red cells, and a megakaryocyte are seen. Wright-Giemsa stain; reduced 40% from mag. × 900.

megakaryocytes were seen. The lymphocytes, although decreased, were still present in good number (50.8%). The neutrophilic polymorphonuclear cells were strongly positive for alkaline phosphatase (2+-4+) (Fig. 2).

Histology.—The architectural landmarks were everywhere retained. Congestion was mild. Sinusoidal spaces were easily discernible. The most striking change was an extensive and diffuse, though occasionally uneven and spotty, hypercellularity of the red pulp due to actively proliferating hematopoietic tissue. The latter comprised elements of all series. More primitive components predominated within the pulp cords where in some areas they formed compact sheet-like aggregates. They were accompanied by myelocytes and more mature granulocytes, as well as numerous megakaryocytes and a diffuse sprinkling of normoblasts. The megakaryocytes formed small groups of three to six cells (Fig. 4) or lay singly.

The lymph follicles, which were inconspicuous on the gross examination, were small and widely separated (approximately one per two to three low-power fields).

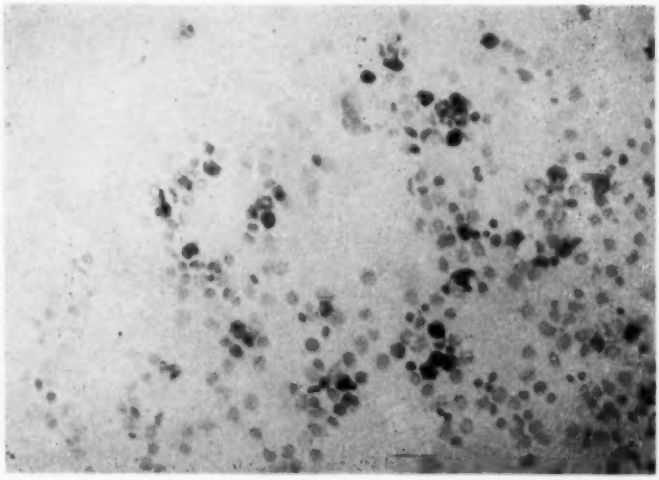

Fig. 2 (Case 1).—Splenic imprint. Several strongly positive neutrophilic polymorphonuclear cells are present. Alkaline phosphatase stain (Gomori-Takamatsu) with neutral red counterstain; reduced 40% from mag. × 700.

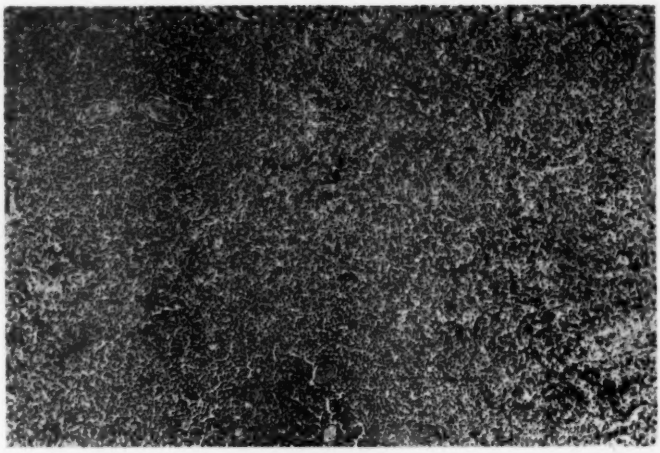

Fig. 3 (Case 1).—Spleen section. Normal-appearing lymph follicles are seen in the left upper field and in the bottom center. Red pulp shows infiltration with myeloid elements. Hematoxylin and eosin; reduced 35% from mag. × 100.

One to four follicles were seen per low-power field of the control spleens. The follicles were nonetheless distinct (Fig. 3), and, though devoid of germinal centers, they were accentuated by central fibrinoid deposits.

A silver impregnation and a connective tissue stain showed a patchy increase both in quantity and in thickness of the reticulum fibers (Fig. 5*A* and *B*) and to less extent of the collagen fibers. The alkaline phosphatase stain showed the usual presence of the enzyme in the vascular endothelium and in the perifollicular region. In addition, ill-defined cells also gave a strongly positive reaction (Fig. 6).

Similar changes were seen in the red pulp of the accessory spleen; however, the follicles were more numerous and conspicuous. Extramedullary hematopoiesis was also present in lymph nodes from the splenic hilum and in the representative specimen of the liver. The architecture of these tissues was intact, and the hematopoiesis was comparable to that in the spleens.

CASE 2 (alkaline phosphatase score 86).

The spleen weighed 2,655 gm. Splenic imprint showed a striking number of nucleated red cells (37.2%). The granulocytes were not significantly increased, but immature forms were present (blast cells

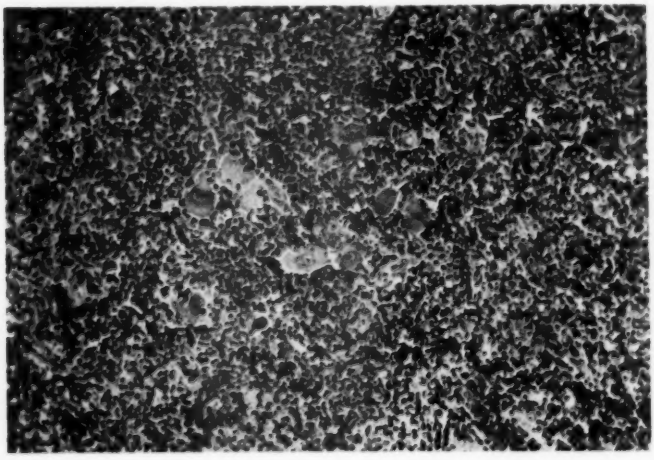

Fig. 4 (Case 1).—Spleen section. Groups of megakaryocytes between other myeloid elements. Hematoxylin and eosin; reduced 35% from mag. × 250.

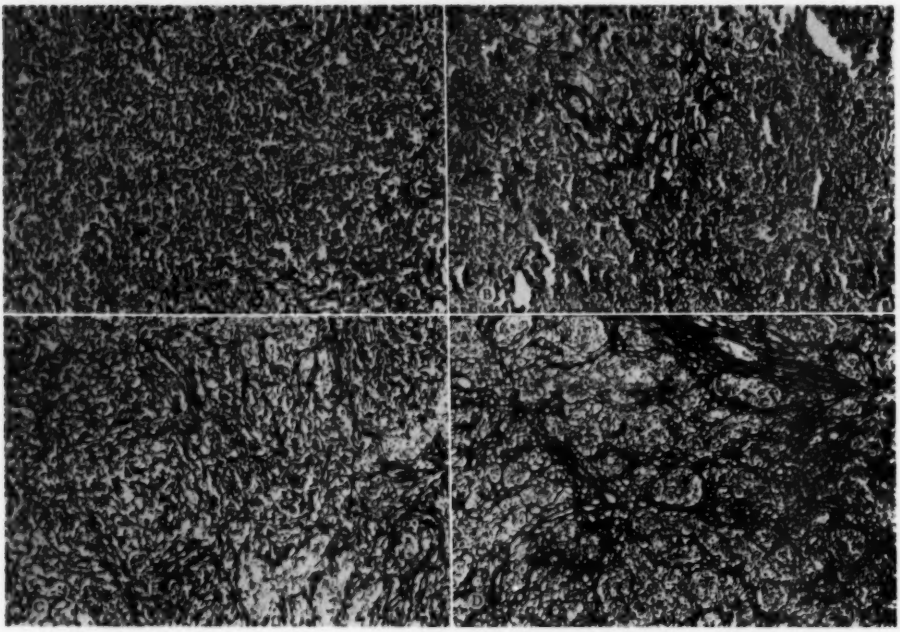

Fig. 5.—*A*, normal spleen, red pulp (for comparison). *B* (Case 1, myeloid metaplasia Type I), patchy increase of reticulum. Individual fibers are thickened. *C* (Case 3, myeloid metaplasia Type II), diffuse increase of reticulum with thickening of individual fibers. *D* (Case 4, myeloid metaplasia Type II), striking increase of reticulum fibers which surround foci of myeloid cells. Individual fibers are very coarse. Silver stain for reticulum (Gordon and Sweet); reduced 55% from mag. × 250.

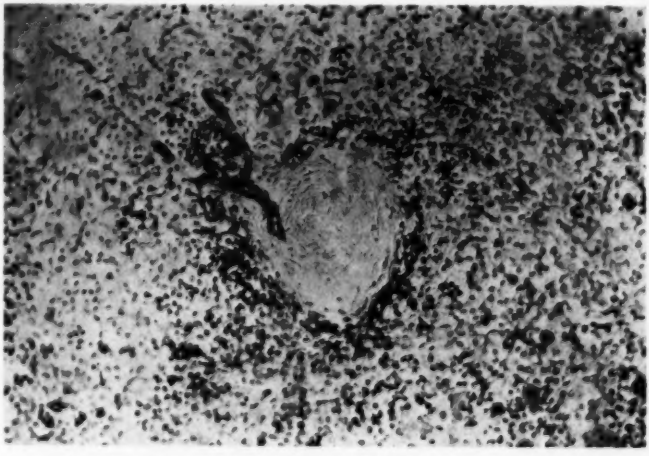

Fig. 6 (Case 1.)—Spleen section. In addition to vascular endothelium and the perifollicular zone, which are normally positive, large numbers of cells are strongly positive for the enzyme. Alkaline phosphatase stain (Gomori-Takamatsu) with neutral red counterstain; reduced 35% from mag. × 250.

1.2%, myelocytes 3.2%). Occasional megakaryocytes were seen. The lymphocytes were decreased in number but still plentiful (48%). Many of the neutrophilic polymorphonuclear cells were positive for alkaline phosphatase (0-2+).

Histology.—The basic anatomic pattern was retained, but the sinusoidal network was somewhat obscured by congestion and fresh blotchy hemorrhages. Moderate hemosiderosis and occasional Gandy-gamma bodies were present, indicating old hemor-

rhages. There was a prominent and diffuse extramedullary hematopoiesis in which the nucleated red cells, mainly at the normoblast stage, were most conspicuous. Blast cells, myelocytes, and megakaryocytes were also present but less numerous. Lymphoid aggregates were present, but they were very small and widely separated (one per low-power field).

Reticulum and fibrous tissue stains showed a mild to moderate diffuse increase with patchy accentuations. Alkaline phosphatase stain gave the usual positive reaction of the vascular endothelium and perifollicular zone, but otherwise it was inconclusive.

Myelosclerosis in this patient was detected nine years before splenectomy. At that time sternal trephine biopsy showed an over-all hypercellularity of the marrow due to a striking focal overgrowth of the granulocytic cells and megakaryocytic elements coupled with an early but definite myelofibrosis. Erythropoiesis was diminished. The bony trabeculae were not appreciably sclerotic.

The patient eventually died of septic complications following surgery for granulomatous endometritis and myometritis. At autopsy an underlying chronic tuberculous process was found. The marrow space of the sternum, vertebrae, and ribs was greatly reduced by extensive osteosclerosis. Scanty blood-forming elements were present among the highly vascular fibrous tissues. Among the remaining marrow elements numerous megakaryocytes and normoblasts stood out. Extramedullary hematopoiesis, largely erythroid and megakaryocytic, was also identifiable in the liver and lymph nodes. This was less conspicuous than in the spleen and was nonleukemic in pattern.

Myeloid Metaplasia, Type II

CASE 3 (alkaline phosphatase score 18 and 9).—The spleen weighed 2,000 gm. Splenic imprint showed a marked decrease in the number of lymphocytes (24%). Nucleated red cells were present in considerable numbers (35.2%). Cells of the granulocytic series were also increased, and both blast cells and myelocytes were prominent (blast cells 6%, myelocytes 7.6%). Reticulum cells, many with phagocytized material, were increased. This was most likely due to the large number of blood transfusions the patient received during the previous three years. Only scanty megakaryocytes were present. Almost all neutrophilic polymorphonuclear cells were negative for alkaline phosphatase.

Histology.—The red pulp was the seat of diffuse hematopoiesis. The nucleated red cells were numerous, and megakaryocytes were present in moderate numbers, but myelocytes were inconspicuous. Small clumps of blast cells were present throughout the organ. Mature granulocytes were scattered throughout. There was moderate congestion with old and recent hemorrhages, Gandy-gamma bodies, and diffuse and focal fibrosis. There was a widespread accentuation of reticuloendothelial elements by hemosiderosis.

The lymphoid tissue was extremely sparse and atrophic (one small follicle per three to five low-power fields).

The reticulum stains showed a very striking diffuse increase of reticulum fibers, the individual fibers being considerably thickened (Fig. 5*A* and *C*). There was also a patchy slight diffuse increase of collagen tissue. Alkaline phosphatase stain, apart from the positive reaction in the vascular endothelium and perifollicular region, was inconclusive.

The lymph nodes from the splenic hilum showed reticuloendothelial hyperplasia and striking overgrowth of megakaryocytic elements. Myelopoiesis and erythropoiesis were inconspicuous. Lymphoid tissue was hypoplastic. Bone marrow biopsies were done on two occasions. One and one-half years before splenectomy, the marrow was hypocellular and showed marked myelofibrosis. Eight months after splenectomy, it showed granulocytic hyperplasia and small foci of myelofibrosis.

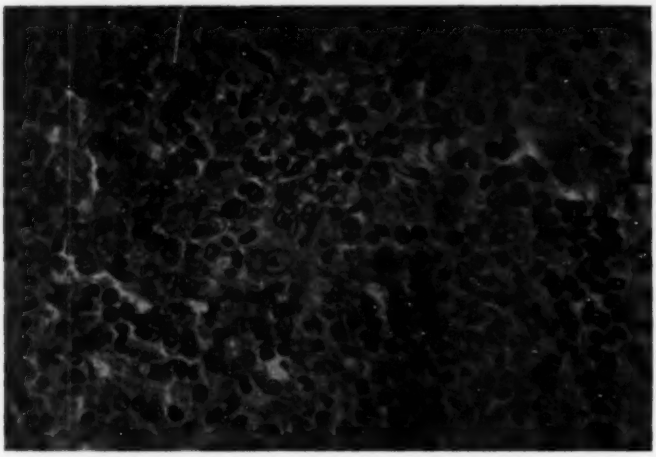

Fig. 7 (Case 4).—Spleen section. Predominant cells are myelocytes and blasts. Only scanty polymorphonuclear cells, nucleated red cells, and megakaryocytes are seen. Hematoxylin and eosin stain, high-power view; reduced 35% from mag. × 700.

Case 4 (alkaline phosphatase score 16). The spleen weighed 3,000 gm. Splenic imprint showed a remarkable increase of cells of the granulocytic series. The myelocytes were predominant (40%), but blast cells were also considerably increased (6.4%). The lymphocytes were diminished in number (20%), and nucleated red cells were rather infrequent. Only a few megakaryocytes were present. Almost all neutrophilic polymorphonuclear cells were negative for alkaline phosphatase.

Histology.—The architectural pattern of the spleen was extensively and diffusely modified by an overgrowth of marrow elements coupled with congestive and hemorrhagic changes as well as with widespread fibrosis. Lymphoid follicles were completely replaced. The predominant proliferating elements were the cells of the granulocytic series at all levels of maturation. Numerous blast cells arranged in small clumps or cords were seen (Fig. 7). Cells in mitosis were frequent. Most of the infiltrate was present in the red pulp cords. Sinuses were inconspicuous. Megakaryocytes were numerous, but nucleated red cells were rare and largely found within the sinuses.

The reticulum stain revealed a striking increase of reticulum fibers. These were very coarse (Fig. 5, *A* and *D*) and frequently surrounded the foci of myeloid cells. There was also a slight diffuse increase of collagen tissue. Apart from vascular endothelium, the alkaline phosphatase stain was inconclusive.

Biopsy of the otherwise intact liver revealed occasional megakaryocytes in the sinusoids.

The other stains did not contribute to the differentiation of the two types of myeloid metaplasia, but they were helpful in recognition of some cellular elements.

Summary of Findings

The following differences were apparent between the spleens of patients, who had myeloid metaplasia with high and with low alkaline phosphatase content of mature neutrophils:

1. The cases with low alkaline phosphatase (Type II) had a considerable decrease of lymphocytes in splenic imprints when compared with those of Type I. This finding was corroborated by a greater decrease, both in size and in number, of lymph follicles in histological sections of these cases.

2. There was a much greater proportion of primitive granulocytes (blast cells and myelocytes) in Type II myeloid metaplasia.

3. There was a greater over-all change in the architecture of the spleens of Type II, especially in the spleen of Case 4.

4. Coarse reticulum fibers were more striking and much more diffusely increased

in Type II cases, while they were only patchily increased in Type I. The differences in the fibrous tissue were less striking.

5. The majority of neutrophilic polymorphonuclear cells were positive for alkaline phosphatase in Type I, while only a very occasional cell was positive in Type II. This correlates well with the scores in the peripheral blood. Histologic sections were inconclusive in this respect.

No striking differences were observed in numbers or in maturity of the cells of the erythroblastic series or in megakaryocytes.

Comment

The differences in the splenic pathology in two types of myeloid metaplasia were essentially those of degree and not of kind. In Type II (low alkaline phosphatase of neutrophils) the proliferative lesion was more marked and the degree of immaturity of the white cells more striking. It seemed that the whole process was more advanced and possibly of longer duration. This type (Type II) may represent the very end of the myeloproliferative process, which not infrequently starts with frank polycythemia vera (panmyelopathy) and later, when proliferation of other mesenchymatous cells (fibroblasts, osteoblasts, and possibly reticulum cells) comes to the fore, continues under the clinical picture of myeloid metaplasia with myelofibrosis. Finally the process becomes spent, but strangely the white blood elements continue to proliferate even more vigorously and with greater evidence of immaturity, simulating chronic granulocytic leukemia.

Alkaline phosphatase of neutrophils is always high in polycythemia vera. It tends to decrease as the fibrotic and sclerotic process advances, and finally it may become very low. It almost seems to form an index of the progression of the fibrotic and sclerotic process.

Oechslin [11] has shown that the process of myelofibrosis and myelosclerosis is a progressive one. It spreads centrifugally; e. g., when central bones like the sternum, vertebrae, and pelvis become fibrotic and later sclerotic, an unusual erythropoietic activity takes place in the long bones where, in adult life, the bone marrow is hematologically nonactive and consists of fat. But these sites, too, eventually become fibrotic and sclerotic until finally very little, if any, erythropoiesis takes place in the entire bone marrow. Our studies confirm these observations. In cases we considered transitional, i. e., with features of both polycythemia vera and myeloid metaplasia (full bone marrow, large spleen with evidence of extensive myeloid metaplasia on splenic aspiration, and normal or low hematocrit and hemoglobin values), the bone marrow of the tibia obtained by surgical trephination showed marked hyperplasia of all three cellular elements of the blood. These cases were always associated with very high alkaline phosphatase values of neutrophils (e. g., score 228). On the other hand, in a case of myeloid metaplasia with advanced fibrosis of sternal and pelvic marrow whose alkaline phosphatase, although still elevated, was considerably lower (86), the tibial biopsy revealed fibrotic and fatty marrow. It might be assumed that cases of myeloid metaplasia with low alkaline phosphatase scores will also have inactive and fibrotic tibial marrow. This was actually the case in Case 3 of the present study.

There is a great similarity between the classical pathologic picture of the spleen in chronic granulocytic leukemia and the spleen of our Case 4. However, this patient had all the features of myeloid metaplasia. Both clinical and hematological data were those of myeloid metaplasia and not of chronic granulocytic leukemia. The patient even had a possible history of polycythemia vera. Bone marrow and liver specimens did not show leukemic changes. Furthermore, the alkaline phosphatase score was 16, a low normal value and definitely outside the leukemic range. Sixteen cases of chronic granulocytic leukemia which we examined previously [2] had extremely low scores rang-

ing from 0 to 4, with an average of 0.8. Thus our Case 4 falls distinctly outside the cytochemical range of leukemia. In spite of all this, the spleen is still remarkably like that of chronic granulocytic leukemia. It is very possible that the so-called typical leukemic picture of the spleen may occur in certain cases of myeloid metaplasia. Whether these cases eventually develop a full-blown picture of chronic granulocytic leukemia with involvement of other organs one cannot as yet say.

Summary

Surgically removed spleens of four patients with myeloid metaplasia were examined cytologically, histologically, and histochemically. Two of them were removed from patients who had high alkaline phosphatase levels of the mature granulocytes and two from patients with low levels of this enzyme.

The following differences were noted between these two groups: Spleens of patients with low alkaline phosphatase of granulocytes showed greater cytological immaturity, especially in the granulocytic series; greater distortion of the splenic structure, with almost complete disappearance of the lymph follicles, and a striking increase of the reticulum fibers and of fibrous tissue. It is concluded that the cases with low alkaline phosphatase of the granulocytes are more advanced in the process of myeloproliferation. From a morphologic-cytochemical point of view some of them are similar to the cases of chronic granulocytic leukemia, but, as they have entirely different initial clinical courses, it is thought that a similar or almost identical pathologic picture may be present in two different but related processes, chronic granulocytic leukemia and myeloid metaplasia.

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Biological Studies of Dihydrocholesterol

IV. Effect of Bile Acids and Other Choleretic Agents on Dihydrocholesterol-Induced Cholelithiasis in the Rabbit

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Introduction

Previous studies have shown that the feeding of dihydrocholesterol to rabbits consistently leads to inflammation of the biliary tract and deposition of concrements in the bile ducts and gallbladder.[1] It was further demonstrated that the formation of these concrements, which consist largely of bile salts, could be prevented entirely or in part by the simultaneous administration of dehydrocholic acid.[2] These results suggested that the experimentally induced cholelithiasis could be affected by changes in either the pH of the bile or the rate of bile flow. Since the administration of dehydrocholic acid produced no significant changes in the pH of the bile,[1,2] it was pertinent to study the effect of naturally occurring bile acids (cholic acid, deoxycholic acid, hyodeoxycholic acid) and other choleretic agents (dehydrocholic acid, florentyrone [Zanchol], Gallogen, Dreole and MA-C2 *) upon the development of the dihydrocholesterol-induced cholecystitis and cholelithiasis and upon the tissue concentrations of cholesterol and dihydrocholesterol.

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* The chemical formulas and the manufacturers of these compounds are listed in Table 1.

Methods

Eighty-nine male chinchilla type or albino rabbits were maintained for four weeks on the different dietary regimens indicated in Table 1. The animals were kept in individual cages and had access to food at all times. The basic diet was Purina Rabbit Chow Pellets to which olive oil, U. S. P., had been added to obtain a final concentration of 12% olive oil. When required, the food was treated with dihydrocholesterol, bile acids, or both, as described previously.[1,2] The choleretic drugs were thoroughly mixed with the food, together with the olive oil, by use of a mechanical mixer.

At the end of four weeks the animals were killed by intravenous injection of pentobarbital. During this time the animals had either maintained their weight or gained weight. There were no obvious toxic effects attributable to any of the choleretic drugs tested, although the dosages used were as high as or higher than those known to produce adequate choleresis in acute animal experiments[4,6] † or in clinical trials.[7]

The severity of the cholelithiasis was estimated by measurement of the dry weight of the concrements present in the gallbladder and by histologic examination. The concentrations of cholesterol and dihydrocholesterol in liver, muscle, and serum were determined at the end of the experimental period. The histologic and biochemical methods[1,2] and the procedure for estimating the pathologic involvement of the biliary tract[3] have been described previously.

Results

Table 1 summarizes the average weight of the rabbits at the start of the experiment, the average dry weight of the gallstones, and the average pathologic involvement of the 16 groups of animals used in this study.

† None of the drugs used in this study had been tested in rabbits. Charlier and Vandersmissen[8] administered single doses of Dreole amounting to 300 mg. per kilogram to guinea pigs, without observing harmful effects.

TABLE 1.—*Degree of Cholelithiasis and Pathologic Involvement of Rabbits*

Group	Dietary Additions: Dihydrocholesterol, 0.5%	Dietary Additions: Choleretic Agent, 0.25%	Animals, No.	Weight of Animals, Kg.	Dry Weight of Gallstones: Average, Mg.	Dry Weight of Gallstones: Range, Mg.	Pathologic Involvement (Arbitrary Units)*
A	+	None	18	2.7	180	0-360 †	10.4
B	—	None	7	2.3	0	—	0.9 ‡
C	—	Deoxycholic acid	4	2.9	0	—	2.5
D	+	Deoxycholic acid	6	2.5	0	—	3.3
E	—	Cholic acid	3	2.1	0	—	1.7
F	+	Cholic acid	4	2.1	0	—	2.8
G	—	Dehydrocholic acid	5	2.1	0	—	1.0
H	+	Dehydrocholic acid	10	2.6	10	0-90	5.1
I	—	Hyodeoxycholic acid	5	3.4	0	—	4.0
J	+	Hyodeoxycholic acid	6	3.2	270	0-460 §	7.3
K	—	Gallogen ‖	3	2.3	0	—	0.7
L	+	Gallogen ‖	4	2.7	150	20-290	6.7
M	+	Zanchol ¶	4	2.8	220	30-580	9.3
N	+	MA-C2 #	4	2.8	220	0-330 §	9.0
O	+	Dreole **	6	2.1	100	0-270 ††	4.5

* For method of estimation see Reference 3.

† One animal in this group had no gallstones.

‡ Three rabbits in this group showed an increase in periportal cellular infiltration. The gallbladders and extrahepatic bile ducts were normal.

§ One animal in this group had a rudimentary gallbladder containing no concrements.

‖ Gallogen (Massengill) is the diethanolamine salt of mono-(+)-camphoric acid ester of 2, 4-dimethylbenzyl alcohol.

¶ Zanchol (Searle) is γ-oxo- γ-fluoranthoylbutyric acid.

MA-C2 (Ames) is β-[4(1,2,3,10b-tetrahydrofluoranthoyl)]-propionic acid.

** Dreole (Lilly) is p-hydroxyphenylsalicylamide.

†† Two animals in this group had no gallstones.

All but 1 of the 18 animals fed 0.5% dihydrocholesterol for four weeks (Group A) had gallstones. On the basis of previous studies, a 100% incidence of cholelithiasis was expected in this group. It is not known why one of the rabbits proved resistant.

The gross appearance of the biliary tract of animals fed 0.5% dihydrocholesterol for three weeks has been described in an earlier paper.[2] The present control group (Group A) had received 0.5% dihydrocholesterol for four weeks and had lesions of similar

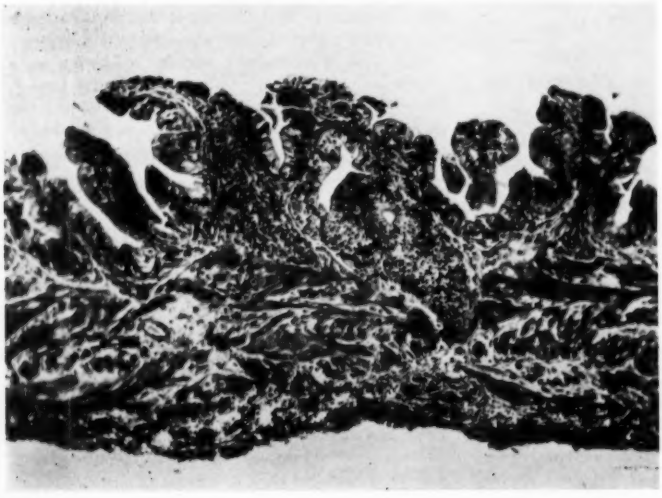

Fig. 1.—Gallbladder of a rabbit fed 0.5% dihydrocholesterol for four weeks (Group A). Stones weighing 203 mg. (dry weight) were present in the fundus. The mucosal folds are thick and adherent. Cellular infiltration of lymphocytes and histiocytes extends throughout the wall. Both the lamina propria and serosa are thickened by fibrous tissue. Hematoxylin and eosin; reduced 10% from mag. × 100.

severity. The 17 animals of this group which developed gallstones (average dry weight 180 mg.) showed the expected congestion and thickening of the walls of the gallbladder. In about one-half of the animals the intra- and extrahepatic ducts contained concrements.

The histologic changes in this group were similar to those described in animals receiving 0.25%-1% dihydrocholesterol for three to six weeks [1-3] and consisted briefly of edema, fibrosis, and cellular infiltration of the biliary tract (Fig. 1).

On the average, control Group A had the greatest pathologic involvement, 10.4+. This value is very close to that of 11.5+ obtained when 1.0% dihydrocholesterol was fed for four weeks.[3]

The results listed for Group H (0.5% dihydrocholesterol plus 0.25% dehydrocholic acid), in which 8 of 10 animals were found to be free of gallstones, are likewise in accord with an earlier observation [2] that dehydrocholic acid inhibits or prevents the formation of gallstones. However, in the present experiment the inflammatory reaction was severer than that reported in the previous study. This is probably due to the fact that in the present experiment the animals received dihydrocholesterol plus dehydrocholic acid for a period of four weeks, i. e., for one week longer than in the earlier study.

The data of Table 1 further show that Zanchol, MA-C2, Gallogen, and hyodeoxycholic acid were ineffective in suppressing the inflammatory manifestations and gallstone formation. The gallbladders of the animals receiving dihydrocholesterol plus 0.25% of these substances (Groups J, L, M, and N) could not be distinguished from those of Group A. The lowered pathologic involvement observed in Groups J and L was due to the absence of calculi from the intra- and extrahepatic bile ducts, suggesting that these agents did exert some choleretic effect.

In contrast, deoxycholic acid and cholic acid suppressed the development of the inflammatory lesions and completely prevented the formation of concrements (Groups D and F) (Fig. 2). These two naturally occurring bile acids proved to be even more effective than dehydrocholic acid in preventing the dihydrocholesterol-induced irritation of the biliary tract.

The six animals receiving Dreole plus dihydrocholesterol (Group O) had an average pathologic involvement of 4.5+; only four of these animals had gallstones (average dry weight 100 mg.). Both the weight of the gallstones and the over-all pathologic involvement were distinctly diminished in comparison with the control group receiving dihydrocholesterol alone. The lowered pathologic involvement resulted largely from suppression of the inflammatory reaction of the biliary tract in animals receiving dihydrocholesterol plus Dreole (Fig. 4).

Hyodeoxycholic acid fed without dihydrocholesterol was found to be an irritant of

Fig. 2.—Gallbladder of a rabbit fed 0.5% dihydrocholesterol and 0.25% cholic acid for four weeks (Group F). No calculi were found in the biliary tract. The mucosal folds are thin. The lamina propria is delicate. Only an occasional lymphocyte is present. The histology is that of a normal gallbladder. Hematoxylin and eosin; reduced 10% from mag. × 100.

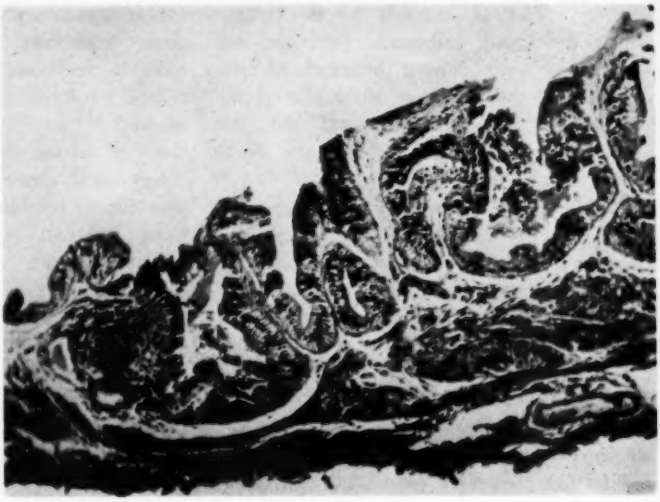

Fig. 3.—Gallbladder of a rabbit fed 0.25% hyodeoxycholic acid without dihydrocholesterol for four weeks (Group I). The mucosal folds, the laminal propria, and the muscularis are edematous. Scattered lymphocytes and histiocytes are present throughout the wall, suggesting that this cholanic acid exerted an irritating effect. Hematoxylin and eosin; reduced 10% from mag. × 100.

the biliary tract in that all five animals of Group I (0.25% hyodeoxycholic acid) showed a slight inflammation without calculus formation (Fig. 3).

Table 2 lists the initial serum cholesterol concentrations and the average serum, liver, and muscle sterol concentrations of the 16 groups of animals at the end of the four-week feeding period. In the present study some increases in serum cholesterol levels were observed in several groups of experimental animals. These increases were most pronounced in the rabbits receiving deoxycholic acid with or without dihydrocholesterol but were also observed in the rabbits fed dihydrocholesterol plus cholic acid, dehydrocholic acid, Zanchol, or Gallogen. There have been reports that cholic acid, but not deoxycholic acid, can produce an increase of serum cholesterol concentrations in cholesterol-fed rabbits.[11] The present study indicates that deoxycholic acid is considerably more effective in producing elevated serum sterol levels than cholic acid, even when the animals were maintained on dihydrocholesterol-free diets (Group C). In addition, deoxycholic acid feeding produced increases of liver sterol concentrations both with and without the addition of dihydrocholesterol to the diet (Groups C and D).

As observed previously,[2,3] the administration of dihydrocholesterol led to the replacement of about one-quarter to one-third of

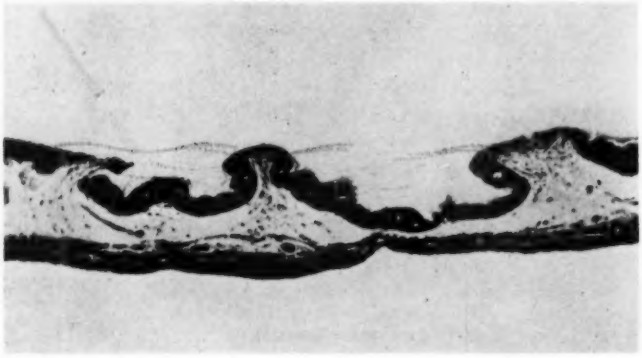

Fig. 4.—Gallbladder of a rabbit fed 0.5% dihydrocholesterol and 0.25% Dreole for four weeks (Group O). The fundus contained concrements weighing 214 mg. (dry weight). The mucosal folds are somewhat flattened due to distention of the gallbladder by bile and concrements. There is edema of the lamina propria but little cellular infiltration. Hematoxlin and eosin; reduced 10% from mag. × 100.

TABLE 2.—*Tissue Sterol Concentrations of Rabbits When Killed*

	Dietary Additions		Initial Serum Cholesterol, Mg. %	Final Serum Sterol			Final Liver Sterol			Final Muscle Sterol	
Group	DHC,* 0.5%	Choleretic Agent, 0.25%		Total Sterol, Mg.%	Cholesterol, Mg.%	DHC in Total Sterol, %	Total Sterol, Mg/Gm.	Cholesterol, Mg/Gm.	DHC in Total Sterol, %	Total Sterol, Mg/Gm.	DHC in Total Sterol, %
A	+	None	46	184	90	51	6.88	3.48	49	0.63	32
B	—	None	42	51	48	6	3.31	2.81	15	0.62	19
C	—	Deoxycholic acid	62	164	145	12	4.82	3.94	18	0.58	3
D	+	Deoxycholic acid	48	291	170	42	18.2	7.72	58	0.66	33
E	—	Cholic acid	40	85	81	5	3.44	2.87	17	0.65	14
F	+	Cholic acid	42	196	101	48	7.87	3.93	50	0.72	42
G	—	Dehydrocholic acid	74	97	97	0	3.11	2.83	9	0.68	19
H	+	Dehydrocholic acid	39	206	94	54	5.81	2.72	53	0.62	31
I	—	Hyodeoxycholic acid	35	47	40	15	2.99	2.72	9	0.62	20
J	+	Hyodeoxycholic acid	28	92	37	60	7.63	3.38	56	0.66	41
K	—	Gallogen †	56	48	50	0	3.84	3.21	16	0.62	23
L	+	Gallogen †	32	149	77	48	5.82	2.86	51	0.55	31
M	+	Zanchol ‡	24	186	92	51	5.16	2.87	44	0.63	33
N	+	MA-C2 §	34	128	66	48	5.31	2.79	48	0.67	39
O	+	Dreole ‖	46	125	73	42	4.14	2.29	45	0.73	26

* DHC indicates dihydrocholesterol.
† Diethanolamine salt of mono-(†)-camphone acid ester of 2,4-dimethylbenzyl alcohol.
‡ γ-oxo- γ-fluoranthoylbutyric acid.
§ β-[4(1,2,3,10b-tetrahydrofluoranthoyl)]-propionic acid.
‖ *p*-hydroxyphenylsalicylamide.

muscle sterol by dihydrocholesterol. None of the choleretic agents studied produced additional increases in either the concentration of dihydrocholesterol or the total sterol in muscle tissue.

Comment

The major cholanic acid constituents of rabbit bile are deoxycholic and cholic acid, with the former predominating. These two bile acids were effective in preventing dihydrocholesterol-induced cholecystitis and cholelithiasis. Dehydrocholic acid, the triketo derivative of cholic acid, inhibited gallstone formation fairly well (8 of 10 animals had no cholelithiasis) but did not protect the biliary tract against the irritating effect of dietary dihydrocholesterol. The other cholanic acid tested was hyodeoxycholic acid (3α,6α-dihydroxycholanic acid), the major bile acid of hog bile. This bile acid produced an irritation of the biliary tract of the rabbit, even when no dihydrocholesterol was administered (pathologic involvement 4.0+), and had little or no protective effect when fed together with this sterol. The failure of hyodeoxycholic acid to inhibit the formation of biliary calculi can be ascribed to the fact that this bile acid is relatively ineffective in promoting the flow of bile.[8,9] Three of the four non-bile acid choleretics tested did not inhibit the experimentally induced cholelithiasis, although the dosages used were generally as high as or higher than those recommended in the literature.[4-7] The failure of Zanchol and MA-C2 to exert significant protective effects was disappointing, because these substances are fluoranthenes, the structure of which bears a superficial similarity to that of the phenanthrene nucleus of the steroids. The results obtained with rabbits fed Dreole (*p*-hydroxyphenylsalicylamide) plus dihydrocholesterol suggest that this compound provided some protection against the irritating effect of dihydrocholesterol. Since Dreole is a salicylate, it may well possess anti-inflammatory properties, as well as choleretic activity.

The present investigation confirms the results of earlier studies indicating that the concentration of dihydrocholesterol in the tissues bears no simple relationship to the intensity of the lesions produced in the biliary tract. In fact, while the animals receiving deoxycholic acid plus dihydro-

cholesterol (Group D) had serum and liver sterol concentrations which were much higher than those found in any other group, these animals had no biliary calculi and exhibited minimal inflammation of the biliary tract. This finding suggests that there are two possible explanations for the absence of biliary calculi in rabbits receiving dihydrocholesterol plus cholic, deoxycholic, or dehydrocholic acids in their diet.

1. The administration of bile acids inhibits the conversion of sterols to cholanic acids by the liver.[10] This would explain the accumulation of sterols in the livers of rabbits fed dihydrocholesterol plus bile acids (particularly deoxycholic acid) and would also account for the fact that the conversion of dihydrocholesterol to bile salt-like substances is inhibited. This hypothesis would further serve to explain why the species-specific cholanic acids proved to be the most effective in preventing the formation of biliary calculi.

2. Deoxycholic, cholic, and dehydrocholic acids are more effective choleretic agents than the other substances studied.

If the second explanation is found to be valid, dihydrocholesterol-fed rabbits would prove useful for the evaluation of choleretic drugs. Presumably, a choleretic drug of the same order of activity as deoxycholic acid or cholic acid should inhibit the formation of biliary calculi and the development of the inflammatory reaction. It is important to point out that in such comparative studies the dosages used would require detailed investigation.

Summary

The effect of cholanic acids and other choleretics upon the biliary tract of dihydrocholesterol-fed rabbits was investigated. It was shown that deoxycholic acid and cholic acid completely prevented the dihydrocholesterol-induced cholelithiasis, while dehydrocholic acid was slightly less effective. Hyodeoxycholic acid and three of the four non-bile acid choleretics tested did not suppress the formation of biliary calculi or the development of the inflammatory reaction of the biliary tract; however, Dreole (*p*-hydroxyphenylsalicylamide) partially protected the biliary tract of the rabbits against the irritating effect of dihydrocholesterol.

The dihydrocholesterol was supplied by the Schering Corporation. Deoxycholic, cholic, and dehydrocholic acids were supplied by the Ames Company, Inc., and hyodeoxycholic acid by Canada Packers, Ltd., and the Wilson Laboratories. Eli Lilly & Company, G. D. Searle & Co., the S. E. Massengill Company, and the Ames Company, Inc., supplied the choleretic drugs used in this study.

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Endocrine Function of a Heterotransplantable Human Embryonal Carcinoma

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Introduction

We have recently reported on Pitt 61, an embryonal carcinoma of the human testis heterotransplanted to cortisone-treated hamsters.[6] Some of the animals with grafts of Pitt 61 had an associated uterine hyperplasia, and their tumors required 30% less time to attain transplantable size than those associated with small uteri.[7] At that time, neither the hormone responsible for the large uteri nor the role of the tumor in the production of the hormone had been identified.

Experiments, here recorded, prove that this heterotransplanted human embryonal carcinoma, Pitt 61, produces chorionic gonadotropin in approximately 20% of animals with grafts. Further, the tumor responds to high levels of environmental chorionic gonadotropin by an increase in growth rate.

Materials and Methods

Pitt 61, the embryonal carcinoma used in these experiments, is now in its 48th generation in cortisone-treated hamsters, covering a period of three years. Aside from a 50% increase in the growth rate and an increased prominence of cell membranes there have been no alterations associated with its long-term growth in the heterologous hosts.

The method of transplantation was adapted from that of Lutz et al.,[4] and the continuous cortisone treatment of the hamster hosts, 2.5 mg.* at the time of grafting and biweekly thereafter, was patterned on Patterson's modification[5] of the regimen first described by Toolan.[8] In some of the experiments hypophysectomized hamsters were used for transplantation. They were treated in a manner identical to the tumor-bearing intact hamsters and tolerated the experimental conditions, including the cortisone treatment, with no untoward effects. In other experiments the hamsters were oophorectomized or orchiectomized, usually on the day prior to grafting but occasionally on the day of grafting. In some of these experiments the tumor-bearing hamsters received endocrine therapy in the following daily doses: (1) Chorionic gonadotropin, 100 I. U. (Squibb Follutein)*; (2) Follicle-stimulating hormone, 5 R. U. (Rat Units) (Squibb pituitary gonadotropin).* The controls received only the vehicle in which the hormone was normally suspended.

The ovaries and uteri of the tumor-bearing cortisone-treated hamsters were used as built-in indicators of endocrine alterations of the hosts, and they were weighed separately on a Roller-Smith balance after being dissected free of adjacent fat. In order to distinguish between normal variations in uterine size and a true uterine hyperplasia for a given system we used the statistical fact that on the average only one observation in a population of 378 exceeds the mean plus three times the standard deviation.[2] In applying this rationale to our experiments, we calculated the mean uterine weight plus three standard deviations for the control system under consideration. If a significant number of uteri in the appropriate experimental group weighed in excess of this value it was concluded that they were receiving hormone stimulation in excess of control values. In other words, the value obtained by adding to the mean of the controls three times the standard deviation served as an arbitrary division between normal variations

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* Dr. F. K. Heath, Research Division, Merck & Co., Inc., Rahway, N. J., supplied the cortisone acetate, and Dr. E. C. Reifenstein Jr., Associate Medical Director, E. R. Squibb & Sons, Squibb Building, 745 5th Ave., New York 22, supplied the chorionic gonadotropin and follicle-stimulating hormone used in the experiments.

in uterine size, on the one hand, and true hyperplasias resulting from abnormal hormone stimulation, on the other.

Tissues were examined microscopically after fixation in Bouin's fluid, mounting in paraffin, and staining with hematoxylin and eosin or periodic acid Schiff.

Results

The hormone responsible for the uterine hyperplasia in 20% of hosts grafted with Pitt 61 most likely originated either in the tumor graft or in the ovary of the host. In order to define the role of the ovary in the production of uterine hyperplasia, the uteri from a series of 75 oophorectomized animals grafted with Pitt 61 were examined. None was found to be hyperplastic, while 25% of 44 intact animals used as a control series had significantly enlarged uteri, thereby indicating that the hosts ovaries were essential to the reaction.

The presumed gonadotropic hormone responsible for estrogenic production by the ovaries of those hosts with large uteri could originate either in the tumor or in the pituitary of the host. The site of gonadotropin production was determined by analyzing the ovaries and uteri of 99 hypophysectomied hamsters carrying grafts of Pitt 61. The results of this experiment are outlined in Table 1. A hyperplastic uterus for this series of hypophysectomized animals was 58 mg. (a figure calculated by adding to the mean uterine weight of the nontumor-bearing hypophysectomized control series three times their standard deviation).

TABLE 1.—*Ovarian and Uterine Weights of Hypophysectomized Cortisone-Treated Hamsters, Non-Tumor-Bearing and Tumor-Bearing*

	Animals Analyzed, No.	Tumor Wt., Gm.*	Ovary Wt., Mg.*	Uterus Wt., Mg.*
Non-tumor-bearing controls	26		7.4 (1.4)	35.5 (7.4)
Tumor-bearing				
Normal uteri	78	0.59 (0.43)	7.5 (2.8)	41.6 (10)
Hyperplastic uteri †	21	0.56 (0.29)	11.7 (5.3)	87.8 (36.3)

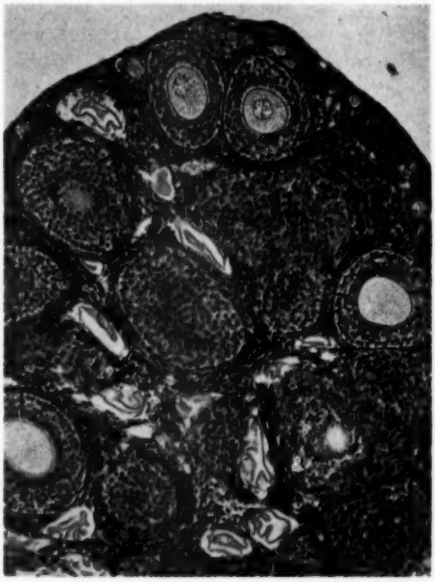
* Standard deviations in parentheses.

† Hyperplastic uteri weighed in excess of the mean + 3 × standard deviation of controls = 58 mg.

Fig. 1.—The ovaries and uterus from an animal bearing a gonadotropin-producing Pitt 61 above, with those from a nontumor-bearing control below.

When this figure (58 mg.) was used as the division between normal and hyperplastic uteri, 21 of the 99 animals in the experiment were found to have uterine hyperplasia. Thus, the tumor graft in the absence of the host pituitary produced a gonadotropic hormone causing uterine hyperplasia through its action upon the host ovary with the same frequency as in intact hosts. In order to be sure that the pituitaries were completely removed from the hypophysectomized hosts, the pituitary fossa was examined at each

Fig. 2.—The ovary from a hypophysectomized cortisone-treated hamster; × 140.



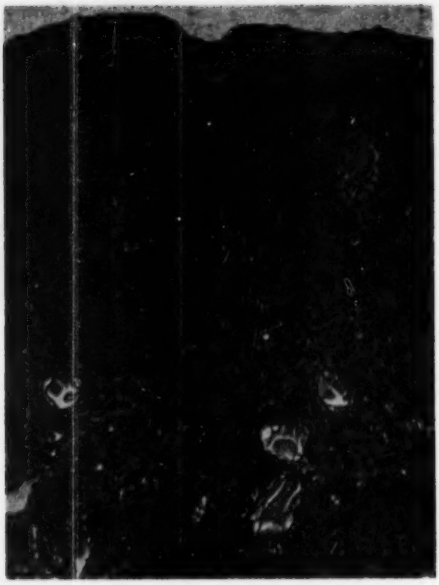


Fig. 3.—The ovary from a hypophysectomized cortisone-treated hamster bearing a hormone-secreting embryonal carcinoma Pitt 61. Notice the hyperplasia and hypertrophy of the stromal cells of the ovary; × 140.

autopsy. Any tissue remaining in the fossa plus that of the hypophysectomy scar was removed, serially sectioned, and examined histologically for a pituitary remnant. Two non-tumor-bearing controls and three experimental animals were found to have pituitary tissue and were withdrawn from the experiment.

The ovaries of the hypophysectomized hosts bearing gonadotropin-secreting embryonal carcinomas were examined in order to identify the type of gonadotropin produced by the tumor (Fig. 1). The ovaries of the hypophysectomized non-tumor-bearing control animals showed atrophy of the stromal cells of the ovary, resulting in an apparent increase in the number of immature follicles (Fig. 2). The ovaries of hypophysectomized hosts with functional tumors were significantly larger than their counterparts not associated with hormone production (Table 1). Histologically these ovaries showed marked hypertrophy of ovarian stromal cells (Fig. 3). In addition, some of the follicles were luteinized, but there was no increase in their number nor other evidence of their maturation. This alteration of the ovary was considered compatible with that caused by chorionic gonadotropin stimulation and indicated that the gonadotropin, secreted by the embryonal carcinoma, Pitt 61, was in fact either chorionic gonadotropin or a chorionic gonadotropin-like substance.

We attempted to produce a chorionic gonadotropin-secreting strain of Pitt 61 by the selective transplantation of hormone-secreting tumors. Each hormone secreting Pitt 61 was grafted into six recipients; all functional tumors in this first generation were transplanted into six more animals, and so on; but after as many as four generations of this selection the incidence of hormone-secreting Pitt 61 tumors was never increased above 30%. We could never predict which of the animals grafted with Pitt 61 would have functional tumors.

The next logical step in the investigation was to identify the cells of Pitt 61 that were elaborating the hormone. Consequently histologic preparations of hormone-secreting Pitt 61 tumors were compared to their non-hormone-producing counterparts. No constant differences could be discerned, however, with hematoxylin and eosin or periodic acid-Schiff preparations. Two cell types could be distinguished in both functional and nonfunctional tumors. One was the typical embryonal carcinoma cell that had the usual characteristic amphophilic cytoplasm and well-stained cell membranes. The nuclei of these cells were large, vesicular, and irregular in shape and contained large densely stained nucleoli (Fig. 4). The second, a dark-staining spindle-shaped cell type, was often found at the margins of clumps of the first type of cells. The nuclei of the dark cells were oval to cigar-shaped and lacked both the irregularity in shape and the huge nucleoli that characterized the larger cells. Transitions in pattern could be seen between the two types, indicating that the dark spindle cells, because of their more

mature appearance, were probably derived from embryonal carcinoma cells (Fig. 5). The dark cells were found as frequently in non-hormone-secreting Pitt 61 tumors as in functional ones, and, furthermore, they were constant constituents of Pitt 94 and D_3, two non-hormone-secreting heterotransplantable embryonal carcinomas. Pitt 94, a slow-growing tumor, which takes in less than 25% of animals grafted, is characterized by a predominance of these dark more mature-appearing cells, which raises the possibility that a connection exists between the growth potential of Pitt 94 and the incidence of the dark cells (Fig. 6).

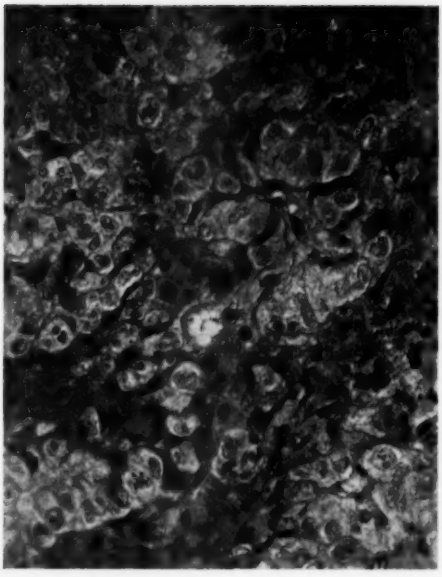


Fig. 5.—A section from a non-hormone-secreting Pitt 61, illustrating the dark cells found in both functional and nonfunctional tumors; × 240.

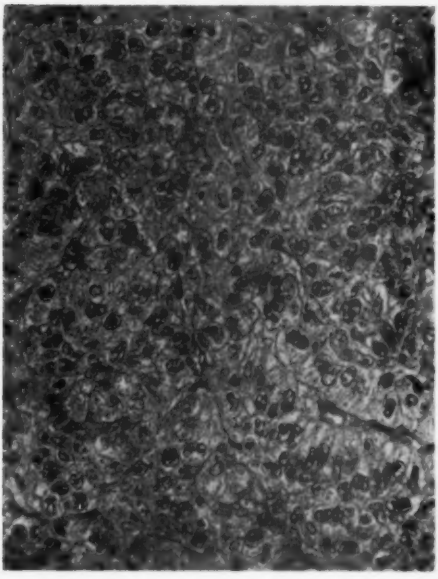


Fig. 4.—Embryonal carcinoma Pitt 61. Notice the typical embryonal carcinoma cells; × 280.

Although no morphologic distinctions could be made between functional and nonfunctional grafts of Pitt 61, the former grew faster than the latter. Reference to Table 2 illustrates that in hypophysectomized hosts functional tumors required 14.9 days to produce the same amount of tumor that nonfunctional tumors required 19.0 days to produce ($P<0.05$). An even greater increment in the growth rate of

Fig. 6.—A section from a Pitt 94 tumor, illustrating the dark cells. Pitt 94 does not produce gonadotropin; × 240.

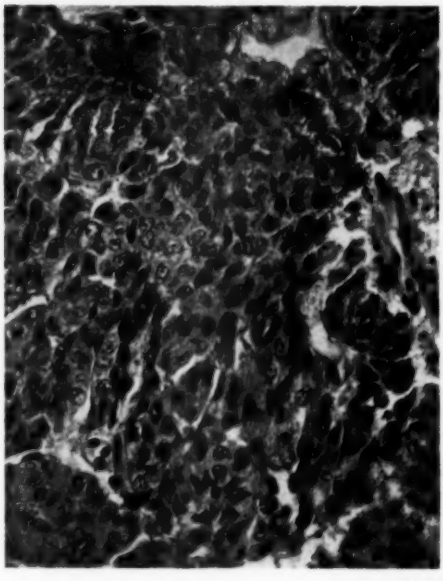


TABLE 2.—*Growth Rates of Pitt 61 Grafted in Hypophysectomized and Intact Cortisone-Treated Weanling Hamsters*

	Animals Analyzed, No.	Time of Tumor Growth, Days *	Tumor Weight, Gm. *	Ovary Weight, Mg. *	Uterus Weight, Mg. *
Intact controls					
Non-tumor-bearing	20			25.5 (3.7)	65 (25.9)
Tumor-bearing gonadotropin-secreting †	17	13.2 (3.0)	0.63 (0.08)	20.8 (6.3)	256.4 (102.5)
Nonfunctional	54	18.6 (5.3)	0.55 (0.09)	21.7 (5.8)	61.7 (30.3)
Hypophysectomized hosts					
Non-tumor-bearing	26			7.4 (1.4)	35.5 (7.4)
Tumor-bearing gonadotropin-secreting ‡	21	14.9 (4.6)	0.56 (0.29)	11.7 (5.3)	87.8 (36.3)
Nonfunctional	78	19.0 (9.3)	0.59 (0.43)	7.5 (2.8)	41.6 (10.0)

* Standard deviations in parentheses.

† Gonadotropin secretion indicated by a uterine weight in excess of the mean + 3 × standard deviation of non-tumor-bearing intact controls = 143 mg.

‡ Mean + 3 × standard deviation of non-tumor-bearing controls = 58 mg.

functional tumors over nonfunctional ones was found when the tumors were grown in intact animals. Gonadotropin-secreting tumors required 13.2 days to attain approximately the same weight in intact hosts for which nonfunctional tumors required 18.6 days ($P<0.001$). The association of hormone secretion and increased growth rate of Pitt 61 tumors raised the question as to whether or not the presence of the hormone itself might not accelerate the rate of tumor growth. Accordingly, an experiment was performed to determine whether exogenous chorionic gonadotropin administered to the hosts would stimulate the growth rate of Pitt 61 grafts. Animals grafted with Pitt 61 were given subcutaneous injections with either 100 I. U. of chorionic gonadotropin per day or 5 R. U. of follicle-stimulating hormone per day. The growth rates of these tumors were compared to that of a non-hormone-treated control series grafted with Pitt 61. Table 3 illustrates that follicle-stimulating hormone-treated animals bore tumors that grew at approximately the same rate as controls, but chorionic gonadotropin-treated animals bore tumors that required only 19 days to produce approximately the same amount of tumor for which the controls required 26 days. These results indicate that exogenously administered chorionic gonadotropin causes a significant increase in the growth rate of Pitt 61 ($P<0.001$).

Although chorionic gonadotropin administration caused an acceleration of growth rate of Pitt 61, no evidence of dependence could be demonstrated upon pituitary or sex hormones. Reference to Table 2 illustrates that hypophysectomy did not influence the growth rate of either functional or nonfunctional tumors. When the growth rate of tumors grown in male hamsters was compared to that of tumors grown in female hamsters, no dependence upon either estrogen or testosterone could be found. Oophorectomy did not influence the growth rate of the tumors.

Comment

The experiments indicate that Pitt 61 produces chorionic gonadotropin in amounts capable of causing significant uterine hyperplasia in approximately 20% of hosts grafted with the tumor. The majority of students of testicular tumors have taken the

TABLE 3.—*Effects of Gonadotropin Treatment of the Host upon the Growth of Pitt 61*

	Animals Analyzed, No.	Time, Days *	Tumor Weight, Gm. *
Controls	49	26 (13.4)	0.64 (0.38)
Follicle-stimulating hormone	37	23 (10.1)	0.63 (0.33)
Chorionic gonadotropin	26	19 (6.1)	0.65 (0.29)

* Standard deviations in parentheses.

stand that when an embryonal carcinoma was found in a patient with elevated serum chorionic gonadotropin elements of choriocarcinoma were most likely hidden either in the primary tumor or in a metastasis. By serial passage of the tumor over the past three years we have been able to examine over 3,000 microscopic sections prepared from some 1,500 grafts of Pitt 61 and have never found areas of choriocarcinoma. As a result we conclude that Pitt 61 is an embryonal carcinoma sometimes capable of secreting chorionic gonadotropin. The presence of an embryonal carcinoma with the function of trophoblast strongly supports the contention of Friedman and Moore [3] and Dixon and Moore [1] that embryonal carcinoma is a stage in the morphogenesis of choriocarcinoma.

We are unable at present to recognize the hormone-secreting cells in the tumor, but the possibilities in this area are not exhausted. Indeed, we know little of the mechanisms that cause hormone production by these grafts. Since by serial passage of the hormone-secreting tumors we have been unable to increase the incidence of hormone secretion over that obtained by passage of random tumors, it does not appear likely to us that hormone secretion is genetically determined by, for instance, mutation. Because each tumor grafted contains a large sample of cells, it might be assumed that each tumor has similar hormone-secreting potential. A stimulus for the activation of this potential causes function in about 20% of tumors. Two possible sources for the stimulus may be postulated. In the one case, a factor originating in 20% of the host animals may initiate endocrine function. In the other case, embryonal carcinoma might represent a stage in the morphogenesis of choriocarcinoma and this ability to secrete chorionic gonadotropin might be interpreted as functional differentiation. The stimulus to produce chorionic gonadotropin in this situation could be considered analogous to an embryonic inductor that would in all probability originate from within the tumor.[9]

Irrespective of the mechanism of activation of hormone function, we have demonstrated that Pitt 61 responds to a high level of environmental chorionic gonadotropin of exogenous, and perhaps endogenous, origin by an increase in growth rate. This parallels the well-known clinical fact that patients with testicular tumors and high levels of circulating gonadotropin have poorer prognoses than those with morphologically similar tumors without endocrine activity.[1] Our evidence would indicate that chorionic gonadotropin stimulates tumor-cell growth, but the mechanism of this growth enhancement is not known.

Summary

Pitt 61, a transplantable embryonal carcinoma of the human testicle, has been shown to secrete a chorionic gonadotropin-like hormone in 20% of heterologous hosts. This finding would support the contention that embryonal carcinoma is a stage in the morphogenesis of choriocarcinoma.

Hormone-secreting tumors grew significantly faster than non-hormone-secretors.

The growth rate of Pitt 61 is stimulated by the administration of chorionic gonadotropin to the heterologous hosts but not by the administration of follicle-stimulating hormone.

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The Cancerigenic Potential of Thermal Injury in the Skin of Whole-Body Irradiated Rats

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Deelman [1] first proposed that carcinogenesis may involve a two-stage mechanism in which the induction of a specific predisposition to neoplasia by an agent such as tar is the first stage and the subsequent precipitation of growing tumor by what would otherwise be a noncarcinogenic stimulus to cell proliferation is the second stage. This conclusion was based on his observation of the development of carcinomas and papillomas at the healing edge of incised wounds in previously tarred skin of mice. Because tumors developed earlier and with greater frequency at the site of healed incisions than they did elsewhere in tarred skin, Deelman concluded that an ordinarily noncancerigenic injury may be cancerigenic at a site specifically predisposed to neoplasia. Other investigators subsequently reported that the two-stage mechanism proposed by Deelman was by no means a constant or predictable phenomenon, and several in fact found that tumors developed in tar-treated skin less frequently at sites of secondary injury than elsewhere.[2,3]

However, MacKenzie and Rous [4] subsequently provided some support for Deelman's two-stage mechanism by observing that a rabbit's ear, after an exposure to tar, benzpyrene, or methylcholanthrene which was of itself insufficient to cause tumor formation, tends to develop papillomas at sites of punch wounds. These investigators stressed the fact that not all injuries are equally effective in provoking tumor development in a specifically predisposed site. Thus when a mustard-acetone mixture was used to promote tumor formation in tarred skin they found that although tumor development was stimulated at sites of mild inflammation it was inhibited at sites of severe inflammations. They also observed that an injury which was effective in promoting tumor development on the previously treated skin on one species of animal was not necessarily effective in another species.

In 1954, Berenblum reviewed the conflicting evidence that had accumulated on this subject.[5] He concluded, first, that the validity of the hypothesis of a two-stage mechanism of cancer development has not yet been satisfactorily confirmed and, second, that even if it should be ultimately confirmed in respect to some tumors there are many circumstances in which it would not be applicable.

Two recent studies in this laboratory led to the performance of the experiments herewith reported. One was the observation by Koletsky and Gustafson that a single total-body exposure to ionizing radiation was capable of predisposing virtually all organ systems of the Wistar rat to tumor development.[6] The other was the development by Ross et al.[7] of a method for producing a standardized circumscribed third-degree cutaneous burn in the rat. If virtually all tissues of the rat are predisposed to tumor development by an appropriate dose of ionizing radiation, it would be of interest to find out whether or not tumors of the predisposed skin might be caused to develop preferentially at the site of thermal injury.

In the studies reported herewith, third-degree thermal burns were produced in the

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skin of rats, which had survived total-body irradiation of 620 r in a single dose, to evaluate thermal injury as a *promoting* agent in the causation of skin tumors.

Materials and Methods

Rats Subjected to Total-Body Irradiation and to Standard Thermal Burns.—One hundred male rats (Wistar) (150-200 gm.) were subjected to 620 r total-body irradiation.* After irradiation, the rats were individually caged in temperature- and humidity-controlled quarters. During the first 30 days after irradiation daily weights and census were taken. Antibiotic therapy (penicillin and dihydrostreptomycin [Combiotic]) was administered on alternate days for the first 21 days to combat intercurrent infection.

Of the 100 rats originally subjected to irradiation, there were 33 survivors at 30 days postirradiation. These animals constituted the experimental group. At 62, 82, 112, 146, 292, and 368 days after irradiation a standard third-degree burn was produced on each animal. Each of the six thermal burns was produced at a designated site on the shaved abdomen of the rat by exposure to a flash-burn exposure at 5 cal. per square centimeter per second for one second.[7] The sites of thermal injury on each occasion were observed by gross inspection and graded by the following arbitrary designations with reference to healing.

I. Initial burn reaction. This was a firm yellow raised plaque measuring 2.5×1.0×0.2 cm.

II. Presence of erythema in the periphery of the burn, associated with a firm center.

III. Presence of central softening and discoloration of the burn site.

IV. Presence of ulceration of the burn site.

V. Presence of secondary necrosis of the base and/or the burn edges.

VI. Presence of granulation and early healing of burn site.

VII. Scarring and cicatrization.

The rate of healing was tabulated for all animals and all burns according to the above scheme.

All rats were weighed at frequent intervals, and gross examinations for skin lesions or palpable masses were done periodically until death of the rats ensued. Only occasional animals were killed, to avoid the occurrence of death of animals over weekends or holidays.

At death, all rats were fixed in formalin *in toto*. After fixation and evisceration, diagnostic skeletal roentgenograms were done. Gross and microscopic sections of all tissues were examined. Each burn scar was excised and fixed in formalin as a separate specimen, and step-serial sections were studied microscopically.

Control Rats Subjected to Standard Thermal Burns Only.—Twenty-four male rats (Wistar) (150-200 gm. each) were subjected to two thermal burns of 5 cal. per square centimeter per second for one second. These rats were maintained under the identical conditions described for the irradiated rats. At intervals corresponding approximately to the irradiation-burn interval at the time of death of the experimental rats, the controls were killed and necropsy and examination of burn scars were carried out as described above.

Standard Thermal Burns.—Flash burns of 5 cal. per square centimeter per second for one second were produced on the shaved abdominal skin of all rats according to a previously described method.[7]

* Irradiation data: 218 kv., 15 ma., 1 mm. Cu, 0.5 mm. Al filters, HVL 1.35 mm., Cu target distance 32.1 cm., 128 r per minute at target. Total r dose of 620 delivered in 4.8 minutes.

TABLE 1.—*Distribution of Tumors in Rats Surviving a Single Irradiation of 620 r*

	Mo. After Irradiation			
	0-6	6-12	12-18	18-21
Rats that died	2	14	13	3
Rats with tumors	1	9	13	3
Total tumors	1	19	25	17
Tumors per rat	1.0	2.1	1.9	5.7

Results

Production of Tumors in Rats As Sequel of Single Exposure to Total-Body Irradiation.—The incidence of tumors in rats as a sequel to a single exposure of midlethal total-body irradiation which was noted by previous investigators was confirmed in the

TABLE 2.—*Distribution and Type of Tumors Arising After 620 r Total-Body Irradiation in Rats*

System	Tumors		
	Benign	Malignant	Total
Skin & subcutaneous	10	18	28
Musculoskeletal	0	2	2
Respiratory	0	3	3
Cardiovascular	0	0	0
Hemic & lymphatic	0	1	1
Gastrointestinal	1	2	3
Genitourinary	8	5	13
Endocrine	9	1	10
Central nervous system	0	2	2
Total tumors	28	34	62
Incidence (rats with tumors)	16/32	20/32	

present studies. In Table 1, the distribution of tumors found in 33 rats at the time of death is presented.

A single tumor was found in one of the two rats that died during the first six months after irradiation. Between 6 and 12 months, 9 of the 14 rats that died had a total of 19 tumors. Beyond 12 months all rats were found to harbor multiple tumors, with an average of 5.7 tumors per rat in the group that died 18-21 months after irradiation.

In addition to demonstrating the increased incidence of tumor development in irradiated rats, these experiments further confirmed the observations of other investigators in the finding that virtually all organ systems were involved by both benign and malignant lesions (Table 2). Thus, a total of 62 tumors was found in the following groups: 26 of the 32 animals were found to harbor tumors; 16 of 32 rats had benign lesions, and 20 had malignant lesions; 13 rats showed multiple tumors of separate origin; 1 rat had six primary tumors, the highest number found. All organ systems were involved, with the exception of the cardiovascular system. The skin and subcutaneous systems were the seat of the largest number; 28 of the 62 tumors were found there.

To evaluate the efficiency of severe thermal burns as a tumor-promoting agent, third-degree burns of the skin were produced at 62, 82, 112, 146, 292, and 368 days after irradiation. A total of 156 flash burns of the skin of rats was produced. The scar at the site of each of these burns was examined at the time of death of the rats. The distribution of burn scars available for study at various intervals after irradiation is presented in Table 3. Twelve burns were a maximum of 3 months old; seventeen were 3-6 months old; eighty-four were 6-12 months old; thirty were 12-18 months old, and fourteen were 18-21 months old.

Healing of Burn Scars.—After each episode of thermal injury the burn was observed daily and evaluated according to the outline presented earlier. Gross evaluation of burns done at 62, 82, 112, 146, 292, and 368 days after irradiation revealed no notable differences in healing time of any of these burns compared to each other or in relation to the healing time of nonirradiated rats subjected to similar burns. All groups, including controls, healed between 12 and 18 days. No gross evidence of tumor was ever noted in a burn scar.

In 10 of the 32 irradiated rats abnormal epithelial changes in burn scars were recognized microscopically. Fourteen of the total of one hundred fifty-six burn scars in these rats showed changes consisting of a minimal degree of squamous-cell hyperplasia in nine scars and focal atypical squamous-cell changes in five scars. In no instance could these changes be recognized as tumor. Of 24 control burns in nonirradiated rats no epithelial changes were noted. In addition, 5 of 32 irradiated rats showed focal areas of squamous-cell hyperplasia not related to the burn scars.

TABLE 3.—*Age of Burn Scars Studied in Relation to Survival of Rats After Total-Body Irradiation*

Time of Thermal Burn After Irradiation, Days	Rats Burned, No.	Burn Scars Studied at Varying Times After Irradiation, No.				
		0-3 Mo.	3-6 Mo.	6-12 Mo.	12-18 Mo.	18-21 Mo.
62	32	0	1	15	8	8
82	32	0	2	18	6	6
112	32	0	3	19	9	0
146	32	3	1	21	7	0
292	17	2	9	6	0	0
368	13	7	1	5	0	0
Total	--	12	17	84	30	14

Comment

There can be little doubt that the irradiated rats were predisposed to tumor development. Twenty-five of the 30 rats that survived irradiation for six months or longer developed one or more tumors, with an average of 2.4 tumors per animal, which is a frequency approximately three times greater than that of spontaneous tumor development in the Wistar strain.[8] That the skin was particularly disposed to neoplasia was indicated by the fact that cutaneous tumors accounted for approximately 50% of the total. Of the 156 burns that were produced in the previously irradiated animals, 128 were followed for six months or longer. Thus, there was ample opportunity for tumors to develop at sites of thermal injury if the latter had any significant propensity for provoking tumor development. Despite this, none of the 156 burn scars which were studied grossly and microscopically disclosed evidence of tumor. In 14 the epidermis covering the healed burns was slightly thicker than was observed in comparable burns in the nonirradiated control group. Occasional atypical cells were encountered in five of these. The finding of similar sites of epidermal thickening, with and without atypical cells elsewhere, in the irradiated rats made it unlikely that these abnormalities could be related to the burns.

There was no evidence that previous irradiation affected the healing time of the burns. The average time of healing of burns in the nonirradiated controls was 14 days. The average healing time of burns in the previously irradiated animals was 14.6 days, with a range between 13 and 18.

Summary and Conclusion

Thirty-three of one hundred rats that survived a single dose of total-body irradiation (620 r) for more than 30 days were followed until death. During the course of their postirradiation course, deep thermal burns of the skin were produced at intervals between 62 days and 368 days after irradiation. Complete autopsy of the animals failed to reveal evidence of tumor formation in any of 156 burn scars, although 62 neoplasms, both benign and malignant, involving all organ systems with the exception of the cardiovascular system were discovered. The results of this study indicate that severe cutaneous burning in a Wistar rat predisposed to neoplasia by total-body irradiation does not result in localization of tumors at the sites of the burns.

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Mechanism of Removal of Fluid and Particulate Material from the Respiratory Tract of the Duck

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The mechanism of absorption of fluids, foreign proteins, and particulate matter from the respiratory tract of mammals has been studied. Robertson,[1] in 1941, after reviewing the literature, pointed out that the principle means employed by the body for the immobilization and removal of particulate matter was phagocytosis by large ameboid cells referred to as alveolar phagocytes or "dust cells of von Innes." These cells enter the lymphatics and pass to the lymph nodes. The rate at which carbon-laden macrophages migrate into the lymph channels is slow. Drinker and Field[2] thought that certain kinds of minute particles passed directly into the lymphatics without the intermediate step of phagocytosis.

In 1903, MacCallum[3] was very much interested in the mechanism of absorption of granular materials from the peritoneum. He commented:

> . . . there has been for a long time a desultory discussion as to the manner in which various materials are absorbed from the peritoneum and even in the case of solutions it does not yet seem quite clear whether they are absorbed exclusively by the lymphatics or very largely by the veins. . . . To explain the passage of these granules through the endothelial wall into the lumen of the lacuna, it seems necessary to suppose that the connections of the endothelial cells are so lax that the violent pumping action of the respiratory movement is enough to force material between them when they come to form the only obstruction to its entrance.

It would seem to be the consensus that direct absorption through the mucosa of the larynx, trachea, and bronchi is slight. Little absorption occurs from the pulmonary alveoli, with the exception of water and simple crystalloids.[4] Colin,[5] in 1873, gave 25 liters of water intratracheally to a horse within a period of three hours without serious consequence. Winternitz[6] and Smith observed that isotonic saline in dogs was absorbed very rapidly from the alveoli. Absorption of foreign serum from the respiratory tract does occur in guinea pigs and dogs; however, the degree of absorption is minimal.[7,8] Drinker et al.[8] showed that horse serum was transferred in the dog directly from the alveoli to the blood.

The anatomic structure of the respiratory system in the duck is similar in many ways to that of mammals. However, there are certain fundamental differences that would suggest a possible variation in function. There are no lymph nodes in the duck. The deep lymphatics of each lung are drained by vessels which emerge with the pulmonary vein. The superficial lymphatics of the lung form a wide network on its lower surface. Vessels emerge from this latter network at its outer edge and join the lymphatics following the internal thoracic artery. These lymphatic channels commence as branches draining the abdominal muscles and the diaphragm. They receive the pulmonary lymphatics and branches from the ribs and intercostal muscles and then communicate with the anterior vena cava.[9]

Air sacs, of course, are characteristic anatomic structures in birds.[10] There are nine air sacs in the chicken and duck and seven in the turkey.[10-12] The total volume of these air sacs in the duck is probably four to five times greater than that of both lungs combined. All of the air sacs in the

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duck communicate directly with the larger bronchi, and some are continuous with the sternum, humerus, and vertebrae.

Little attention has been given to the mechanism of the removal of fluid and particulate matter from the respiratory tract of birds. In the present study isotonic saline, fluorescein sodium, saccharated iron oxide, India ink, liquid petrolatum, and Lipodium spores were put into the trachea of white Pekin ducks. Observations were made to determine how these substances were removed from the respiratory tract.

Methods and Materials

White Pekin ducks varying in age from 1 month to 2 years were used. They were kept in small batteries in the laboratory during the time of the acute experiments and in an outside pen for the longer experiments. Food and water were available to them at all times. The intratracheal injections were made with a syringe and an attached small plastic catheter 5 cm. in length. The mouth was opened manually. When the external larynx was opened for inhalation, the catheter was inserted into the trachea for a distance of 2 to 3 cm. The catheter was quickly withdrawn when the injection was completed. Because the birds were held in an upright position with neck elevated for a brief interval after completing the intratracheal injection, regurgitation was reduced to a minimum.

Isotonic saline was given intratracheally to two ducks. The quantity and the time when given are shown in Figure 1. A total of 320 ml. was given to one duck within an interval of two hours. Two hundred milliliters of this fluid was given within a period of 45 minutes. Six ducks were given a single intratracheal injection of 50 ml. of a 0.5% solution of fluorescein sodium in distilled water. Several samples of blood were removed from the leg veins during the first 15 minutes. Other specimens of blood were removed after 4 to 5 hours and after 18 to 24 hours. The blood was citrated and observed for fluorescence with a high-intensity long-wave ultraviolet light.* Usually 4 ml. of blood was put into a test tube with 4 ml. of a 1.0% solution of sodium citrate. Fluorescence was observed after 12 to 24 hours. Control samples of blood were removed before the sodium fluorescence was given.

Twenty-five milliliters of a saccharated iron oxide solution,† containing the equivalent of 100 mg. of elemental iron, or 4 mg. per milliliter, was given intratracheally to one duck. This bird was killed 60 minutes later. A 12.0% concentration of India ink, 0.5 ml., suspended in liquid petrolatum was given intratracheally to 20 ducks. Five of

* A. S. Aloe No. 52140 ultraviolet mineralight, high-intensity long-wave, 3,660 A.

† Feojectin, Smith, Kline & French Laboratories, Philadelphia.

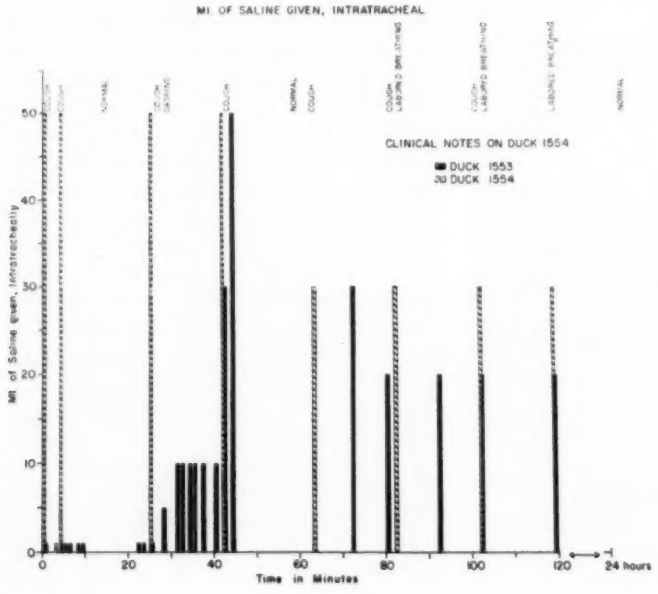

Fig. 1. — Intratracheal injection of saline in the duck. Notice the large amounts given within short intervals of time.

these were killed 5 to 15 minutes later; five, after 48 hours, and ten, after 65 hours. Ten additional ducks were given 0.5 ml. of the same suspension of India ink in liquid petrolatum daily except for Saturdays and Sundays for 10 days. These birds were killed 24 hours after the last intratracheal injection. Two ducks were given intratracheally 25.0 ml. of a 12.0% aqueous suspension of India ink and were killed five days later.

Liquid petrolatum was given intratracheally to 30 ducks. At each injection 0.5 ml. was given. Ten birds were given 10 daily injections except for Saturdays and Sundays and killed 24 hours after the last. Twenty birds were given 7 to 134 intratracheal injections and killed immediately or up to 18 days later.

All ducks in this experiment were killed by severing of the spinal cord. The ribs were cut on each side. The sternum was reflected onto the neck, permitting good exposure of the thoracic and abdominal viscera and the air sacs. This area then was observed under ultraviolet light for fluorescence in the lungs, air sacs, liver, gallbladder, and intestines. The trachea and lungs subsequently were removed intact. After the trachea was opened and the lungs were sectioned several times, all tissues were carefully examined for fluorescence. When the gallbladder was found to fluoresce, the bile was removed and put into the test tubes for further observations.

Sections were removed routinely from the proximal, middle, and lower third of the trachea and from both lungs. Other sections of tissue were removed for histologic study when gross changes were present. All sections were fixed immediately in a 4.0% solution of formaldehyde. Paraffin sections were prepared and stained routinely with hematoxylin and eosin. Select sections were stained with the periodic acid-Schiff reagent and with the Perl stain for hemosiderin. Osmic acid was used for the demonstration of lipids.

Experimental Data

Sodium Chloride in Trachea of Ducks.—These ducks were markedly dyspneic after receiving 100 to 150 ml. of saline (Fig. 1). Sometimes a small amount of fluid was sprayed from the external trachea. The birds soon returned to normal after the intratracheal injections of saline were discontinued. Forty-eight hours later both lungs were moderately hemorrhagic and slightly edematous. No fluid was present at this time in the air sacs (Fig. 2).

Fluorescein Sodium in Trachea of Ducks.—One adult duck was given intratracheally 50 ml. of fluorescein sodium within a period of two to three minutes. There was a minimum of respiratory difficulty immediately, but 10 minutes later the bird appeared normal. Five additional ducks were given intratracheally 50 ml. of fluorescein sodium. The degree of fluorescence in the blood progressively increased after the fluorescein sodium was given; the maximum intensity was reached 8 to 10 minutes after the injection. The blood removed four to five hours after the intratracheal injection of fluorescein sodium showed less fluorescence than the specimens removed after 10 to 15 minutes. There was no fluorescence in the blood that was removed 18 to 24 hours later. There was no fluid present in the air sacs of the ducks that were killed 18 to 24 hours after fluorescein sodium was injected intratracheally. A small amount of fluorescence, however, was

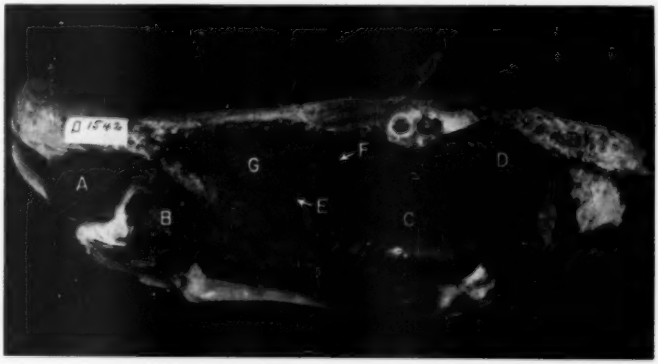

Fig. 2.—Air sacs in the duck after the intratracheal injection of latex. Air sacs: (*A*), cervical; (*B*), clavicular; (*C*), lesser abdominal; (*D*), greater abdominal. (*E*) shows the point of communication between the lung and the lesser abdominal air sac; (*F*) is the area of communication between the lung and the greater abdominal air sac; (*G*) is the lung.

present along the wall of the air sacs in three of the four ducks killed 18 to 24 hours after the intratracheal injection of the fluorescein sodium.

The gallbladder and portions of the small intestines fluoresced in the four ducks that were killed 18 to 24 hours after the intratracheal injection of the fluorescein sodium. When the bile was removed and put into a test tube there was considerable fluorescence. There was no fluorescence either of the bile or of the contents of the loop of the intestines in four normal ducks.

Saccharated Iron Oxide in Trachea of Ducks.—The respiratory tract and the lungs were markedly congested one hour after the intratracheal injection of this preparation of iron. A small amount of brownish-colored fluid was present in the air sacs. Histologic sections stained with hematoxylin and eosin showed a large amount of brown granular material within the lumen of the parabronchi, the air capillaries, and the air sacs. A small amount of this brown-staining material was present in focal areas on the surface of the bronchial epithelium. The stroma beneath the epithelial cells lining the air sacs and the parabronchi was brownish-yellow as a result of the presence of this preparation of iron.

The brown granular material present in the hematoxylin-and-eosin-stained sections stained blue with the Perl reaction for hemosiderin (Fig. 3). A few small blue-staining areas were present within the layers of epithelial cells that line the trachea. The number of such foci was insignificant. Small clumps of blue-staining material were present within the lumen of some of the small lymphatic-like channels in the stroma between the lobules of lung tissue. Sometimes small collections of blue-staining material were present between the endothelial cells lining the lymphatic and vascular channels (Fig. 4).

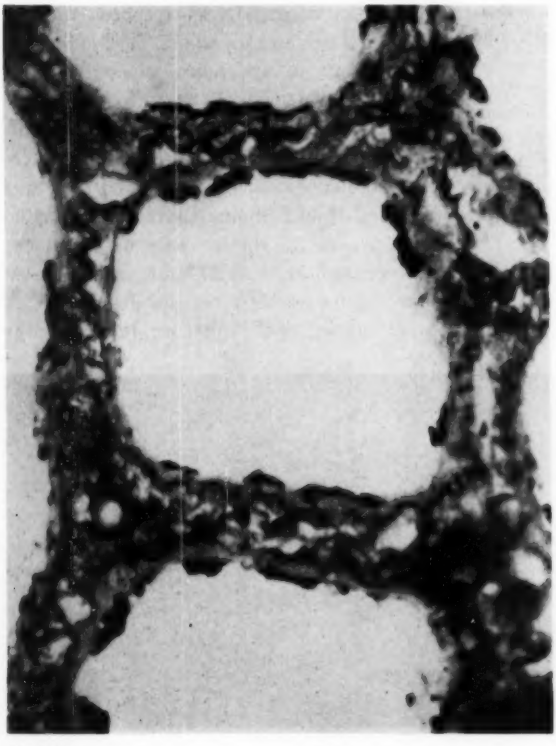

Fig. 3.—Twenty-five milliliters of saccharated iron oxide were put into the trachea an hour before this duck was killed. The iron appears as masses of black staining material on the epithelial surfaces of the parabronchi and in the interstitial tissue. Perl's stain for hemosiderin; × 522.

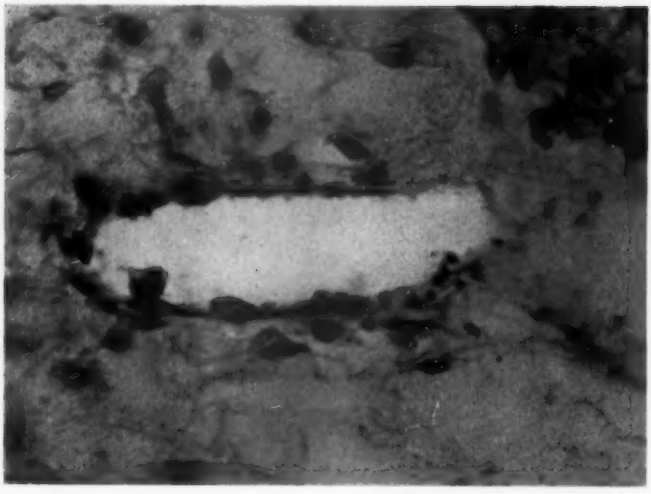

Fig. 4.—Either a capillary or lymphatic in the stroma near a bronchus showing an accumulation of iron staining material between and in the cytoplasm of the endothelial cells. The iron is passing from the bronchus into the circulatory system. Perl's stain for hemosiderin; × 950.

India Ink in Trachea of Ducks.—Carbon particles were present macroscopically in the trachea of each of five ducks and in the lungs of three of the five birds given one injection of India ink suspended in liquid petrolatum and killed 5 to 15 minutes later. Two of this group had carbon particles on the wall of the abdominal air sacs. No carbon particles were observed macroscopically in the respiratory tract of the five birds

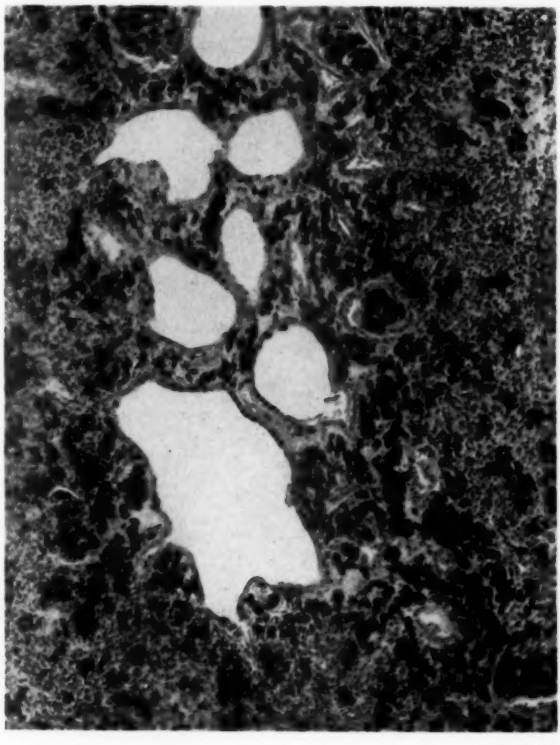

Fig. 5.—Twenty-five milliliters of India ink were put into the trachea five days before this duck was killed. The particles of ink are present in the stroma of the air capillaries and parabronchi. Hemotoxylin and eosin stain; × 150.

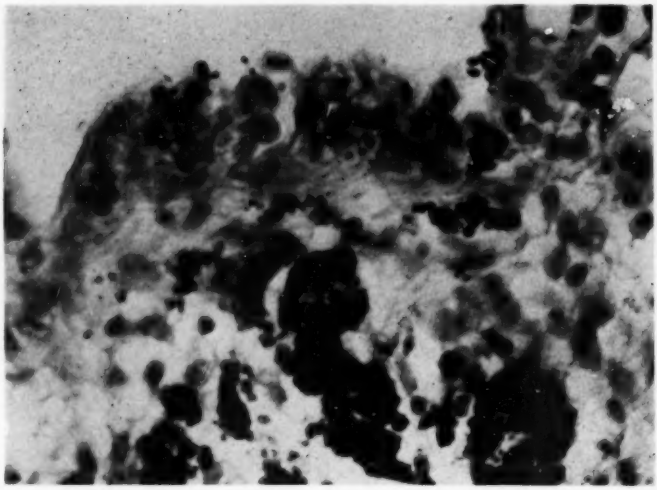

Fig. 6. — Particles of India ink are present between the epithelial cells that line this bronchus. Large masses of carbon particles have collected in the underlying stroma. Hematoxylin and eosin stain; × 260.

given a single injection of the ink in liquid petrolatum and killed 48 hours later. Carbon particles were present macroscopically in the lungs of 4 of the 10 ducks similarly treated and killed 65 hours later.

Two ducks were given intratracheally 25 ml. of the suspension of India ink in distilled water. The birds were killed five days later. Particles of India ink were present in the lumen of the parabronchi and in the

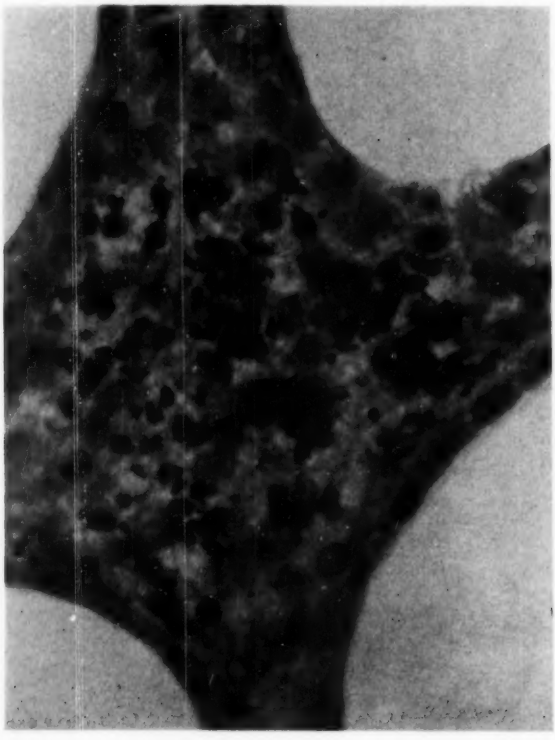

Fig. 7.—Particles of India ink are present in the stroma adjacent to the parabronchi. Hematoxylin and eosin stain; × 1,026.

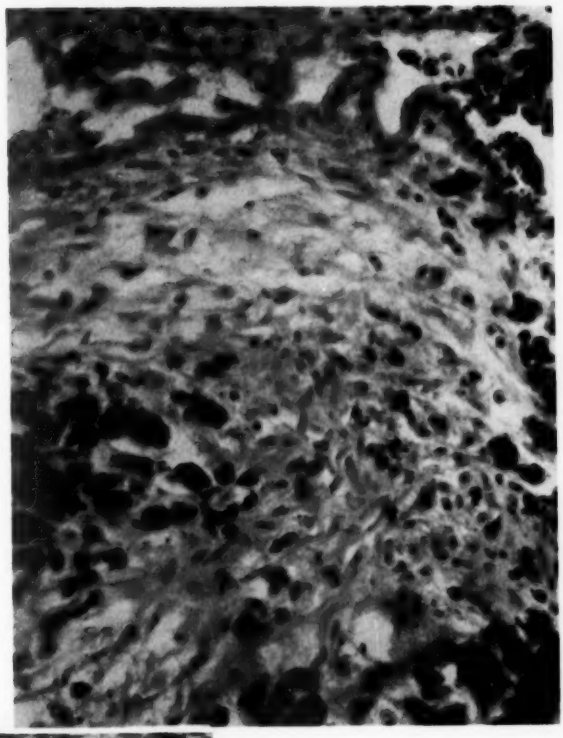

Fig. 8.—Fibroblasts proliferate about the carbon particles in the stroma about a small bronchus. Hematoxylin and eosin stain; × 360.

Fig. 9.—Macrophages phagocytize some of the particles of India ink while they are in the stroma. Hematoxylin and eosin stain; × 665.

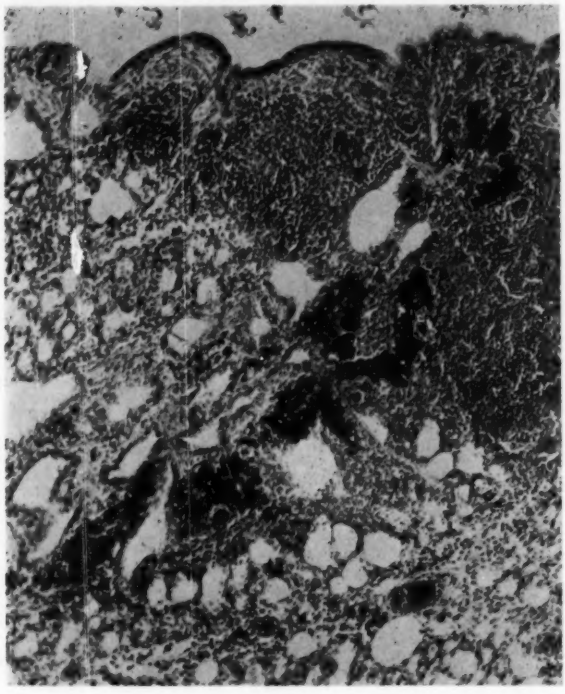

Fig. 10.—Carbon particles concentrated in the groups of lymphocytes. Local proliferation of lymphocytes occurs in the lung after the intratracheal injection of foreign material. Hematoxylin and eosin stain; × 120.

lumen and the walls of the air capillaries (Fig. 5). In many areas, where only a few epithelial cells lined the respiratory tract, particles of India ink were present between the cells (Fig. 6). The particles of India ink, after passing through the layer of epithelium and into the adjacent stroma, formed varying-sized masses (Fig. 7). There was a proliferation of the fibroblasts in the stroma about some of the larger collections of particles of India ink (Fig. 8). Granules of India ink were present in the cytoplasm of some of the fibroblasts. Mononuclear cells, apparently histocytes, also were present in the stroma. Many of these cells phagocytized particles of India ink (Fig. 9). Particles of India ink were present within the group of lymphocytes throughout the lung (Fig. 10).

Ten ducks were given an intratracheal injection of the India ink suspended in liquid petrolatum daily for 10 days. They were killed 24 hours after the last injection. No carbon particles were observed macroscopically in the trachea. Carbon particles, however, were present macroscopically in the lungs of 2 and in the abdominal air sacs of 1 of the 10 ducks. Five of these ten birds did have macroscopic lipid material in the lungs and air sacs. Microscopic study of the respiratory tract of these 10 ducks showed the same distribution of the carbon particles as described above. In some of the larger collections of lymphocytes there was considerable carbon pigment. Extensive areas of acute and chronic inflammation were present in the lower portions of the lungs and sometimes also within the air sacs. A few polymorphonuclear leukocytes and lymphocytes infiltrated the mucosa of the trachea. Few granules of India ink were present in the wall of the trachea. Areas of inflammation occurred in the respiratory tract of ducks given intratracheal injections only of liquid petrolatum similar to that present in the birds given India ink suspended in liquid petrolatum.

Liquid Petrolatum in Trachea of Ducks.—An oily material was present macroscopically in the lungs and in the air sacs of 9 of the 10 ducks given daily injections of liquid petrolatum and killed 24 hours later. There was no macroscopic exudate in the trachea of any of these ducks; however, there was a minimal number of leukocytes and lymphocytes in the wall of the trachea in some of these birds. The lungs, in a majority of the ducks, showed an extensive acute and chronic reaction. A similar reaction was present in the wall of the air sacs.

One of the most interesting lesions observed in the lungs was a local proliferation of lymphocytes. Large groups of these cells were present in the wall of the larger bronchi, and smaller groups of similar cells were present in the stroma between the air capillaries. Sometimes these lymphocytes appeared to develop within the lumen of small vessels, probably in lymph channels (Fig. 11).

Lipid material, as shown by the osmic acid stain, was present in the lumen of the bronchi, parabronchi, air capillaries, and air sacs. Sometimes small globules of lipid-staining material were present within the layer of epithelial cells that line the bronchi. After infiltrating the layer of epithelium that lines the bronchi, these lipid globules accumulated in the stroma in spaces that resembled lymphatics (Fig. 12). Many unidentifiable spaces, some of which may be capillaries, were filled with lipid-staining material. The lumina of some of the small blood vessels were partially filled with lipid material (Fig. 13).

Lipodium Spores in Trachea of Ducks.—Two ducks were given the suspension of Lipodium spores intratracheally and were killed 48 hours later. The lumina of many of the terminal bronchi, the parabronchi,

Fig. 11.—Foci of lymphocytes proliferate in the lungs after the intracheal injection of foreign material. Hematoxylin and eosin stain; × 380.

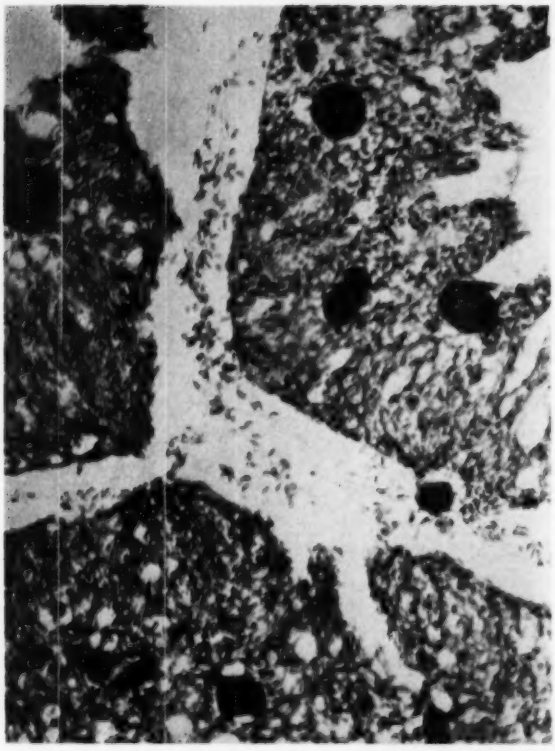

Fig. 12.—Lipid staining material in the lumen of either lymphatics or blood vessels in the interstitial tissue of the lung after the intratracheal injection of liquid petrolatum. Osmic acid stain; × 190.

and the air capillaries were filled with these spores. Sometimes the spores appeared to have adhered to the surface of the epithelium lining the air passages, while in other areas the spores passed through the layer of epithelium and were present in the adjacent stroma (Fig. 14). Sometimes giant cells surrounded these spores (Fig. 15).

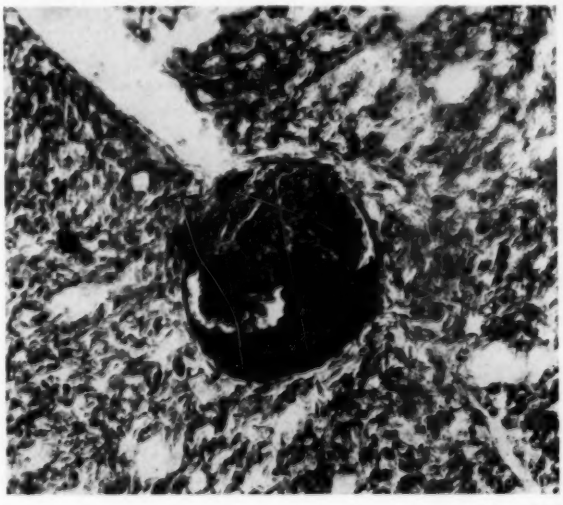

Fig. 13.—The lumen of a small blood vessel in the lung filled with lipid staining material. Liquid petrolatum was put into the trachea 63 hours before this duck was killed. Osmic acid stain; × 280.

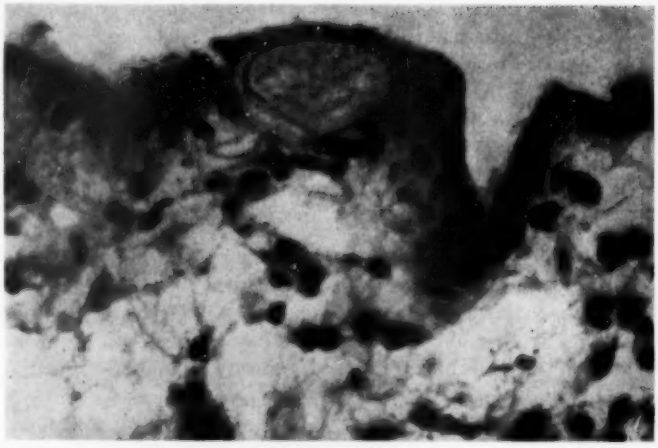

Fig. 14.—A spore in the wall of a bronchus 48 hours after the intratracheal injection of an aqueous suspension of Lipodium spores. Hematoxylin and eosin stain; × 260.

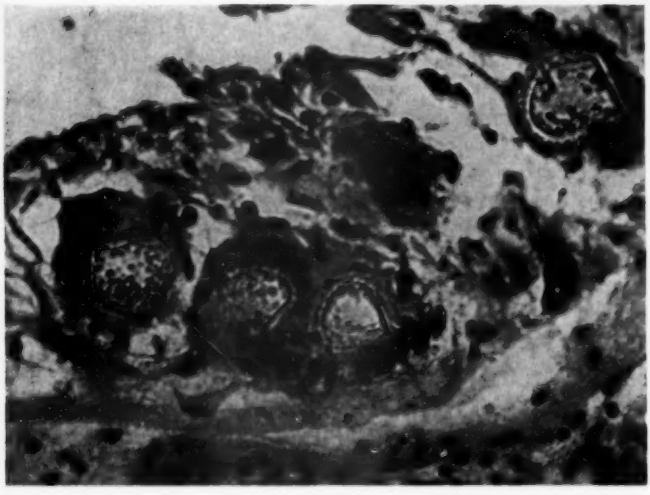

Fig. 15.—Lipodium spores in the stroma beneath the epithelium lining a bronchus. These spores are surrounded by giant cells. Hematoxylin and eosin stain; × 760.

Apparently the spores injured the lining epithelium as they passed from the lumen of the bronchi through the layer of epithelium into the underlying stroma.

Comment

Fluorescein sodium is rapidly transferred from the respiratory tract of the duck into the peripheral circulation. A fluorescent material was present in the blood removed from a leg vein one minute after 50 ml. of 0.5% solution of fluorescein sodium was put into the trachea. It would seem that such a liquid passes rapidly through the layer of epithelium lining the lower portion of the respiratory tract to reach the adjacent stroma. Here it diffuses through the wall of blood vessels and/or lymphatics. The sections removed from the respiratory tract after the intratracheal injection of saccharated iron oxide and stained by the Perl technique for hemosiderin show a large amount of blue-staining material in the stroma and in the wall of some of the smaller vascular channels.

The osmic acid stains made from the tissues of the respiratory tract of ducks given liquid petrolatum intratracheally have many small lipid globules in the layer of epithelium lining the smaller bronchi, the

parabronchi, and the air sacs. In the stroma immediately adjacent to these epithelial structures are large masses of lipid-staining material. Usually it is impossible to say whether these collections of fat are in a lymphatic or a capillary or are free in the tissue spaces. In a few sections small blood vessels did have lipid-staining material within their lumina. The presence of lipid-staining material within the lumen of these vessels would suggest that globules of the liquid petrolatum had entered either a lymphatic that communicated with a blood vessel or had entered directly a blood vessel.

Sections of the respiratory tract from ducks given intratracheal injections of India ink show very nicely that particulate material passes directly through the layer of epithelium lining the air passages to reach the adjacent stroma. Many of the carbon particles remain in the stroma; however, some are phagocytized. Lipodium spores also pass directly through the layer of epithelium lining the respiratory tract in a manner similar to the particles of India ink and the globules of liquid petrolatum.

This study shows that fluids and particulate material leave the respiratory tract by passing between the epithelial cells that line the walls. After reaching the stroma, some of the particles enter the lumen of lymphatics and/or blood vessels by passing between the endothelial cells that form their walls. Some of the particles, while in the stroma, are phagocytized by macrophages. No doubt, these phagocytic cells with particulate material subsequently enter the pulmonary lymphatics and ultimately reach the lumen of blood vessels.

Particulate material in mammals, as is well known, is phagocytized usually within the pulmonary alveolae. These phagocytic cells then enter the pulmonary lymphatics and are filtered out by the regional lymph nodes. In the duck, phagocytosis has not been observed to occur within the respiratory tract. Small particles are phagocytized by macrophages and fibroblasts within the stroma after they have passed the layer of epithelium that lines the air passages. Giant cells surround the larger particles, such as Lipodium spores, in an attempt to phagocytize them. It would seem from this study that much of the particulate material in the respiratory tract of the duck enters the lymphatics and the vascular system directly without first being phagocytized. Some of the foreign particles are retained within the collections of lymphoid cells that develop in the lungs and in the wall of the bronchi.

The air sacs in the duck appear to be the principal area where particulate material is removed from the respiratory tract. These spaces are lined by a single layer of cuboidal or low columnar epithelium. Particulate material, of course, passes such an epithelial barrier more readily than it passes through the layer of stratified epithelium that lines the trachea.

The mechanism of the removal of fluids and particulate material from the respiratory tract of the duck is most interesting when one compares it with the general concept of the local changes that occur in tissues in inflammation. The latter mechanism has been summarized recently by Gozsy and Kato [13] as follows:

> This response is manifested by a suddenly acquired absorptive capacity of the capillary endothelial cells and the whole inner surface of the capillary tube, providing the endothelial cells with a storing and phagocytizing activity. This phenomenon is followed by a progressively increasing permeability of the cement substance, permitting the active transport of damaging particulate matter, previously absorbed on the vascular endothelium, into the perivascular space.

In the removal of fluid and particulate material from the respiratory tract of the duck, the particles pass through the layer of epithelium into the loose stroma where there are lymphatics and capillaries. The particles then pass from the stroma between the endothelial cells into the vascular and/or lymph channels. In this process some of the particles appear to be phagocytized by the endothelial cells that line the channels. Although we have no experimental data to support the suggestion that these phagocytized particles ultimately are released into

the circulating lymph or blood, it would seem most likely that such does occur. The removal of particulate material from the respiratory tract of the duck is essentially the reverse to that which occurs in inflammation. In the latter, colloidal dyes, antibodies, and India ink pass from the lumen of the capillaries into the adjacent stroma, while particles pass from the stroma into the capillaries during removal from the respiratory tract. The local factors that make possible this transfer of particles from the respiratory tract to the circulatory system need additional study.

Summary

In the duck, saccharated iron oxide and fluorescein sodium, when put into the respiratory tract through the trachea, pass directly through the layer of epithelium lining the respiratory tract into the stroma and through the wall of the blood vessels and/or lymphatics. Particles of India ink, globules of petrolatum, and Lipodium spores, when put into the respiratory tract of ducks, migrate through the layer of epithelium and either enter the blood vessels and/or lymphatics directly or are phagocytized within the stroma by fibroblasts and histocytes. The transfer of fluids and particulate materials from the respiratory tract of the duck to the vascular system occurs primarily in the air sacs. The variation in the mechanism of removal of particulate material in the duck and in mammals no doubt is necessitated by the differences in the respiratory and lymphatic systems. There are no lymph nodes in the duck as there are in mammals.

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Intracranial Adrenal Gland

A Case Report

MORRIS F. WIENER, M.D., and S. ALTON DALLGAARD, M.D., Manhasset, L. I., N. Y.

Heterotopic or accessory adrenal tissue has been reported in man in association with practically every organ or structure below the diaphragm since Morgagni's time, in 1740.[1-3] The accessory masses usually are composed of cortical tissue only. Accessory adrenal glands with both cortical and medullary elements are less frequent. Ectopic adrenal tissue associated with the central nervous system has not been reported as far as search of the literature can demonstrate. This communication is a report of such a case An adrenal gland, including both cortex and medulla, was found attached to the leptomeninges of the left frontal lobe and resting on the floor of the anterior cranial fossa.

Report of Case

The patient was a white man, 49 years of age, who was admitted to the service of one of us (S. A. D.) on Oct. 13, 1955. He was in extremis, deeply comatose, markedly cyanotic, and exuding froth from the mouth. The skin was cold and moist; the pulse, irregular at about 60 per minute. Heart sounds were inaudible. The pupils were fixed and dilated. Deep reflexes were absent. He was pronounced dead 20 minutes later.

The history, as obtained from the wife, revealed that the illness began six days previously with persistent and increasingly severe headache. However, he continued at his work of coal-hod carrier. On the third day, he consulted the company physician. On the morning of admission to the hospital, while he was dressing to go to work, the headache became so severe he returned to bed. In a short time he became unconscious and began having tonic convulsions at two- to three-minute intervals. The seizures usually involved the face, body, and limbs and were accompanied by severe sweating, involuntary urination, and bile eructation. They varied in intensity. As the coma deepened, the convulsions diminished.

The patient had indulged moderately in alcohol. There was no history of recent injury, food poisoning, encephalitis, or other neurologic disease.

Treatment consisted of immediate use of oxygen by tent and artificial respiration when respirations ceased.

The clinical diagnosis was cerebral thrombosis or brain tumor.

Necropsy

Necropsy was performed eight hours after death. Only the pertinent findings are given.

The brain is normal in size and shape. The dura mater, falx cerebri, tentorium cerebelli, and falx cerebelli are normal. The leptomeninges show diffuse congestion and edema, with darkly mottled areas more extensive over the base and vertex of the right cerebral hemisphere.

As the brain is lifted, a pancake-shaped mass (Fig. 1) measuring 5.2×3.0×1.8 cm. is found loosely attached to the pia arachnoid of the lateral portion of the inferior surface of the left frontal lobe. Imprint markings are present where

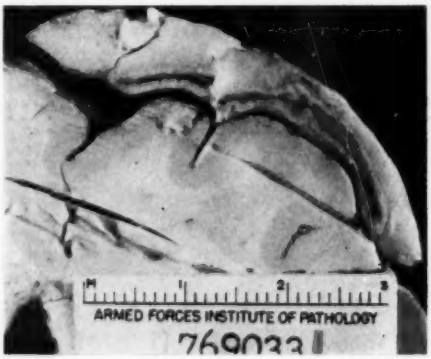

Fig. 1.—Coronal section of left frontal lobe. Lying on the inferior surface, the pancake-shaped mass having the naked-eye appearance of an adrenal gland (AFIP 769033) (AFIP 56 19792-1).

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From the Manhasset Medical Center Hospital.

Presented before the 42d Annual Meeting of the New York State Association of Public Health Laboratories, at Syracuse, May 23, 1958, and before the 34th Annual Meeting of the American Association of Neuropathologists, on June 15, 1958, in Atlantic City, N. J.

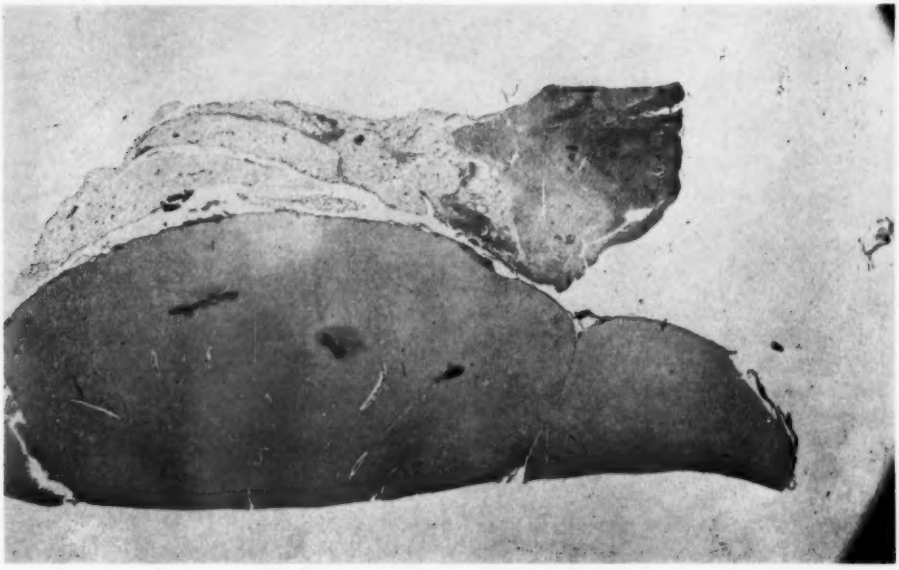

Fig. 2.—Area of attachment of periadrenal and leptomeningeal fat. Artifactitious defect was caused by pressure of pen on slide in ink-siting point of continuity; reduced 40% from mag. ×12 (AFIP 56 19860).

it rested on the floor of the cranial fossa. The consistency is firm, and the appearance is turgid. The brain was placed in 10% formalin fixative for 10 days and then examined by serial coronal sections.

The mass is loosely attached to the leptomeninges and enveloped by a thick layer of normal adipose tissue measuring up to 9 mm. in thickness. The external surface is smooth and slightly lobulated. On gentle sectioning, its attachment to the pial tissue proves very delicate (Figs. 2, 3, and 4). The cut surface reveals a peripheral firm bright-yellow zone measuring on an average 1 mm. in width. The central zone is a dark line. There is nothing sug-

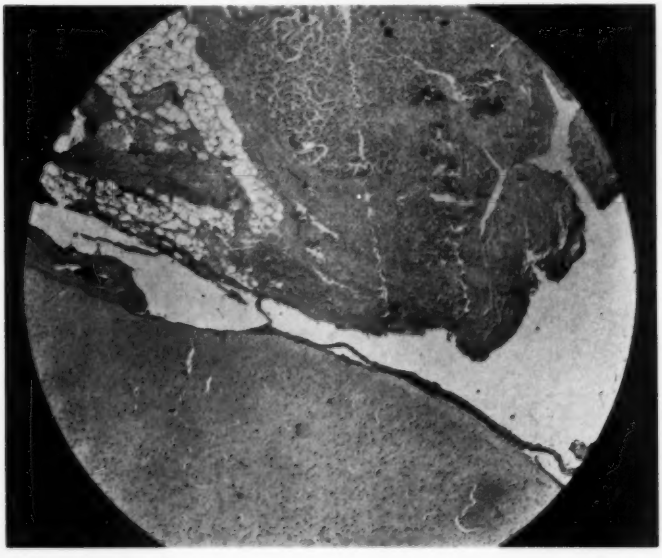

Fig. 3.—Area of attachment; reduced 55% from mag. ×12 (AFIP 56 19859).

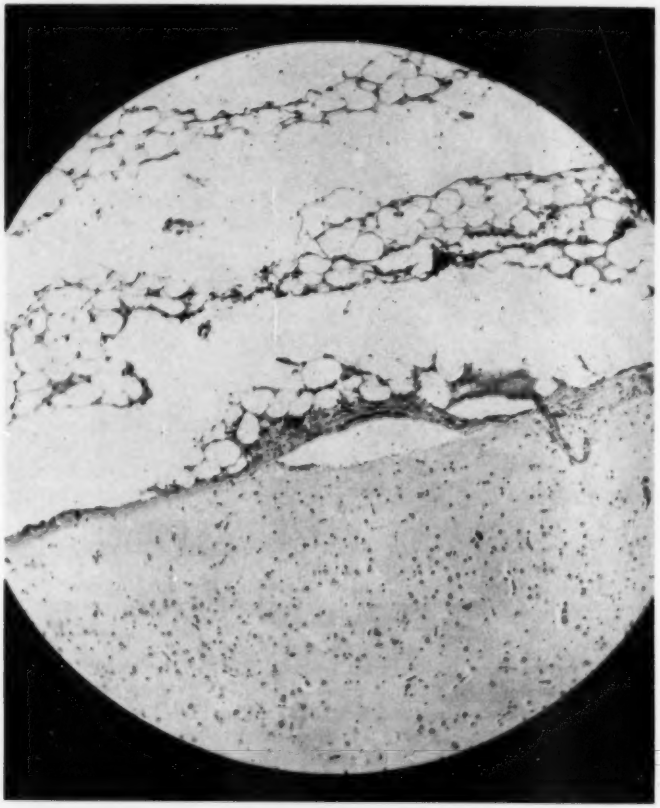

Fig. 4.—Area of attachment; reduced 45% from mag. ×70 (AFIP 56 19856).

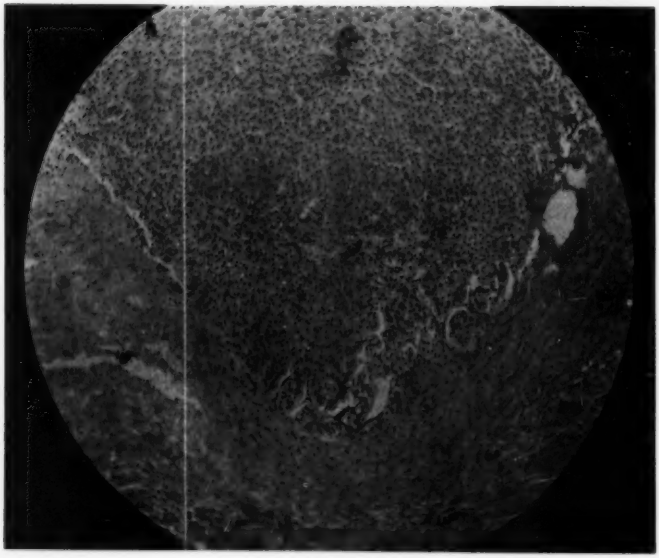

Fig. 5.—Section of the gland showing thick fibrous capsule, cortical zonation, foci of lymphoid cells, prominent vein, and medulla; reduced 55% from mag. ×180 (AFIP 56 19857).

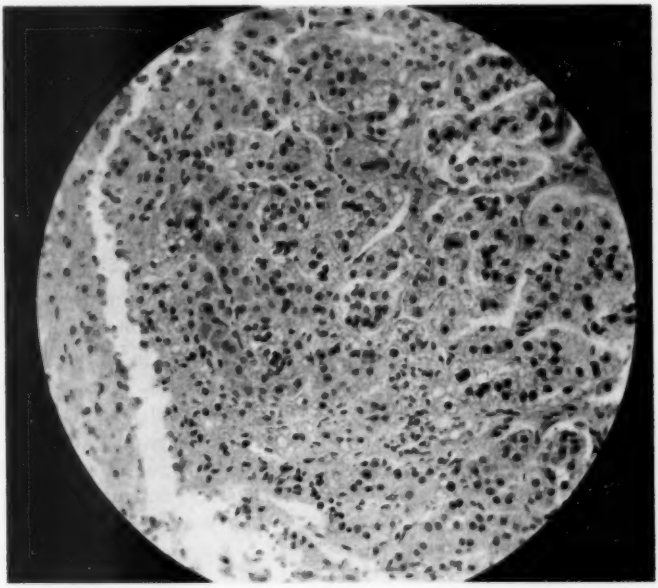

Fig. 6.—Section of the gland showing spongiocytes of the glomerulosa; reduced approximately 50% from mag. × 395 (AFIP 56 19858).

gestive of a vascular pedicle or hilus in the region of attachment. After separation, the pia appears slightly roughened (Fig. 2). The appearance of the mass is indistinguishable from a normal adrenal gland and periadrenal adipose tissue.

Dissection fails to reveal any adrenal glands at the normal retroperitoneal sites.

Microscopy

The mass shows the conventional structural pattern of an adrenal gland with fibrous capsule, cortical zonation, and medulla. The capsule is normal in thickness and composed of mature collagenous tissue. The capsule and surrounding adipose tissue have normal arteries, veins, and nerves, often seen together as a loose triad. The walls of the veins are of normal sturdy thickness, having a spiral arrangement of the longitudinal fibers. Fibrous septa and strands of connective tissue from the capsule penetrate the cortical substance and divide it into indistinct subcapsular lobules. Nodules of cortical hyperplasia are present in the cortex, within the fibrous capsule, and in the pericapsular fat. One of the larger nodules in the periadrenal fat is attached to the fibrous capsule of the gland by a pedicle of connective tissue.

The cortex has three vaguely defined layers—glomerular, fascicular, and reticular. The zona glomerulosa is narrow, and it lies inside the capsule and consists of rounded polygonal or columnar cells packed in ovoid or spherical groups without a lumen. The outer edge of the cell is often seen to adjoin a capillary. Many of the cells are spongiocytes. The nuclei stain deeply (Fig. 6).

The zona fasciculata is the widest of the three zones. The cells are slightly larger than those of the glomerular zone and rich in lipoid droplets, giving the cytoplasm a spongy vacuolated appearance. Both deeply stained and vesicular nuclei are seen (Fig. 5).

The zona reticularis is narrow. It is composed of networks of cells similar to those in the preceding zones, with fewer spongiocytes. In the deeper portion, dark cells containing yellow-brown pigment are present. Light cells are scanty. Groups of cortical cells project into the medulla, making the boundary irregular between the zona reticularis and the medulla (Fig. 5).

The medulla is composed of irregular groups of basophilic ovoid cells which are

in contact with capillaries and venules. The cortical medullary borders show the distinctive tinctorial reaction of the cells of the zona reticularis and the cells of the medulla. The medullary cells lie in a connective tissue network rich in blood supply. Single sympathetic ganglion cells are noted. Collections of small lymphocytic cells are present.

The cells of the nodules are arranged like those of the glomerulosa, in addition to a few cords like the zona fasciculata.

At the junction of the adipose connective tissue and the pia arachnoid (Figs. 2, 3, and 4) there is calcification present as a psammoma body, and there is an irregular plaque of calcium in the slightly thickened fibrosed pia. A large meningeal vessel is seen, with similar caliber to that of a vessel in the adjoining periadrenal fat (Fig. 3).

The extensive intraventricular hemorrhage is found to be of an ordinary type commonly due to lenticular-striate arterial rupture. This cerebral accident is unrelated to the presence of the intracranial adrenal gland.

Anatomical Diagnosis.—The diagnosis was as follows: (1) intracranial adrenal gland and (2) massive intraventricular hemorrhage.

The anatomic continuity of the periadrenal fat and cerebral leptomeninges was verified by Drs. Arthur Purdy Stout, James R. Lisa, Henry L. Jaffe, Paul Klemperer, Silik H. Polayes, Theodore J. Curphey, Hans Popper, Gedeon Eros, and many other competent observers, too numerous to mention, who also examined the sections. Dr. Paul Klemperer vouches ". . . for the location of the mass in the meninges and that it consisted of tissue apparently of the nature of adrenal cortex." Dr. Webb Haymaker, of the Armed Forces Institute of Pathology, examined the sections after artifactitious break in the anatomic continuity; he verified the facts that fatty tissue was present in the meninges in the middle of the gyrus (Fig. 4) and that the large meningeal vessel approximates the vessel in the periadrenal fat (Figs. 2 and 3). He states, ". . . this could be more than mere coincidence." The identity of the histologic minutiae is clearly demonstrable in the area of attachment of the periadrenal fat to the leptomeninges (Figs. 2, 3, and 4).

Comment

The embryogenesis of an adrenal gland within the cranial cavity is the interesting and intriguing aspect of this case.

The adrenal cortex and medulla are entirely unrelated developmentally, histologically, and functionally.[1,2] They are derived from two distinctly different parent tissues and unite only secondarily. The cortex is mesodermal in origin and is recognized in the human embryo at about the fourth week (6 mm.) as a series of buds arising from the celomic epithelium. The medulla is ectodermal in origin and begins differentiation somewhat later in the 18 mm. human embryo from the neural crest and sympathetic ganglia lying near the cortical anlage. It is complete at birth. The medullary cells, which are of sympathochromaffin nature, migrate toward and penetrate the cortical mass along the central vein and become completely enclosed by cortex. The medulla of the adrenal gland is part of a widespread chromaphil system which may be found almost anywhere in the body.[1,2] Unlike the cortex, the adrenal medulla is not essential for life.[3]

The formation of accessory adrenal masses results from fragments of tissue splitting off the cortical anlage. Most accessory adrenals remain near the parent glands, but some become included in or are carried along with other structures which change their position during development. In this manner, accessory glands may appear in almost any region of the abdominal cavity, pelvis, or scrotum. Only rarely do they contain both cortical and medullary elements.

The organogenesis of an intracranial adrenal gland can only be conjectured;

several theoretical mechanisms may be considered. Intracranial adrenal ectopia may result from a misplaced blastomere [4] in which the mesodermal and ectodermal germ layers give rise to the entire gland. In the absence of ectodermal anlage, the medulla can theoretically develop by local differentiation from the ubiquitous sympathetic ganglia. The origin of the thick layer of periadrenal fat attached to the leptomeninges may also be traced to the mesoderm from the misplaced blastomere. On the basis of experimental work by H. T. Blumenthal [7] and by Laqueur and Harrison,[8] conversion of fatty tissue to adrenal cortical cell may be hypothocated. It is recalled that the presence of fatty tissue in the leptomeninges of the frontal lobe is exceptionally rare.[6] Another hypothetical source of mesoderm is suggested by the cephalad position of the pronephros in the early embryo.[10]

The intracranial adrenal organ in our case actually constituted the entire adrenal apparatus in this person. Two factors of embryologic law may explain the absence of any other demonstrable adrenal tissue. It is stated [1] that accessory adrenal tissues undergo atrophy and disappear because they are physiologically unnecessary in the presence of the main or functioning glands. In our case, the intracranial adrenal gland apparently assumed the total adrenal function. Secondly, the absence of adrenals at the normal or accessory sites may be explained by their inhibition or total repression at these sites by the development of a similar functioning organ in another area of the body. Analagous situations are well known. A lingual thyroid, for example, may be found without any trace of thyroid tissue at its usual location.[9]

The location of this intracranial adrenal gland on the floor of one of the anterior cranial fossa happens to be an area of optimal anatomic accommodation. The only evident local tissue reaction was slight fibrous thickening of the leptomeninges and minimal focal calcification. The absence of any local parenchymal reaction in the brain may also be equated with the absolute physiologic dependence of the body upon the vital cortical hormone made available only by the intracranial adrenal gland.

The vascular supply of the intracranial adrenal is meningeal in origin.

Since the specimen had already been fixed in formalin and the adrenal separated from its leptomeningeal attachment to the brain, hormonal assay and angiography were not done.

Summary

A case of an intracranial adrenal gland is reported. As far as can be determined, it is the first instance recorded in the medical literature.

The Armed Forces Institute of Pathology examined the material and prepared the photographs used in this publication.

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Books

Lehrbuch der speziellen pathologischen Anatomie, II. Band 2. Lieferung. Edited by Dr. Martin Staemmler. Price, DM 90.20. Pp. 885, with 303 illustrations. W. de Gruyter, Genthiner Strasse 13, Berlin W. 35, 1957.

This volume, embracing the diseases of the urinary tract in this new and monumental work on systemic pathology, the reincarnated Kaufmann, has been written by M. Staemmler. The microscopic appearance and function of the kidney are reviewed first, following by developmental disturbances and the various pathologic states. Parasites of the urinary tract and kidney traumas are taken up in short chapters. Uremia is likewise treated in a special chapter. Diseases of the ureters, urinary bladder, and urethra constitute the final chapters.

While Staemmler questions the wisdom of the term "malignant" in relation to nephrosclerosis ("a disease of the renal vessels, in principal, can neither be benign nor malignant") he stresses that malignant nephrosclerosis is a morphologically well-characterized entity. He describes the pertinent features as diseased arterioles and prearterioles showing inflammatory and necrotizing changes. The gross picture in general is not characteristic, but dot-like hemorrhages are present in most instances. This classical concept has been recently questioned and the arteriolonecrosis explained as the consequence of the uremia, rather than constituting the characteristic basic lesion in the kidneys. The author gives an interesting and illuminating discussion on the origin of the hypernephroid carcinoma, leaning strongly on its renal origin. In support of this explanation he argues that in view of the fact that benign epithelial tumors in the kidney arising from renal structures are frequent it would be difficult to understand why the common malignant renal tumor should arise from misplaced tissues.

This volume offers 452 good illustrations. It is well written, clear and to the point, and easily understandable. It will be an important aid to the pathologist in the field and to the teacher; the clinician also will find much in this book that will aid in the elucidation of difficult topics. That is, of course, dependent upon his knowledge of German, the language in which the book is written.

It is full of very valuable information which, in such a concise form, is difficult to find and is highly recommended.

OTTO SAPHIR, M.D.

Diseases of the Esophagus. By J. Terracol (Professor of Faculty of Medicine of Montpellier, France) and Richard H. Sweet (Associate Clinical Professor of Surgery, Harvard Medical School). Price, $20. Pp. 682, with illustrations. W. B. Saunders Company, 218 W. Washington Sq., Philadelphia 5, 1958.

This comphensive textbook amply fulfills the need for a modern treatise on diseases of the esophagus. It seems ideally suited for clinicians interested in this organ and medical students. The clinical descriptions are adequate and the surgical techniques with accompanying diagrams excellent. The liberal use of black-and-white photographs and occasional photomicrographs are helpful. It is unfortunate that wider use of color plates could not have been employed. A number of theories pertaining to etiology and pathogenesis are incorporated in some of the chapters. In each instance they have been labeled as such by the authors and should be interpreted as such by the readers. In a book of this scope it is always possible to find something that has been inadvertently omitted. In this instance the description of the employment of the esophagoscope with flexible obturator and description of modern manometric techniques used in investigation of esophogeal physiology and pathophysiology would have added some interesting information to an already excellent work.

Symposium on Cancer of the Liver Among African Negroes; in Kampala, Uganda, August, 1956; Symposium in Leopoldville, September, 1956. Edited by J. Clemmesen. Price, $25 per volume. ACTA, Inc., 5465 Decarie Blvd., Montreal, 1957.

These two reports comprise No. 4, 5, and 6 of Volume 13, 1957, of the Unio Internationalis Contra Cancrum—ACTA, and embody the proceedings of the Symposium on Cancer in Kampala, Unganda, held in August, 1956, as well as the Symposium in Leopoldville, in September, 1956.

The symposium in Kampala was convened under the chairmanship of Dr. Harold L. Stewart and dealt mainly with the problem of cancer of the liver. A review of the meeting would be difficult to present. Pathologists from many parts of the world participated and discussed various aspects of cancer of the liver, particularly with reference to its incidence in Africa. The discussions are included so that the reader can have a good idea of the various points of view.

The symposium in Leopoldville included many of the participants of the symposium at Kampala. The proceedings are essentially a follow-up of those held at Kampala. They are reproduced on excellent paper, with numerous charts, tables, and photomicrographs, and should be of great interest to everyone concerned with the basic problem of cancer of the liver.

A Text on Systemic Pathology. Edited by Otto Saphir, M.D. Price, $32. Pp. 865, with over 800 illustrations. Grune & Stratton, Inc., 381 4th Ave., New York 16, 1958.

This is the first book of a two-volume text to be restricted to systemic pathology. The author's objectives were to provide medical students with more specialized information than is available in the texts of one volume, to provide the practicing pathologist with an advanced reference, and to correlate pathological anatomy with abnormal function to vitalize both. The author invited a few former students and associates to contribute special sections, but most of it is written by himself.

This first volume contains chapters on the heart, vascular system, respiratory tract, mediastinum, urinary system, female and male genital systems, and hematopoietic system. Except for chapters on congenital heart disease (by Maurice Lev), hemaphroditism (by Jay J. Gold), and the hematopoietic system (by Ira Gore), the book was written by the author. The organization of the material in each chapter is conventional, beginning with the congenital anomalies and going through the circulatory disturbances, inflammation, and retrogressive and degenerative changes to neoplasms.

The book is written largely from the author's experience, although references to the literature are numerous. The style of writing is lucid although not always the most compact. The reader is warned in the preface that some of the views might seem unorthodox but that they are based on the author's experience and are his interpretations. The reviewer noted a few errors, which seem to be inevitable in first editions, and some points on which he would disagree. The illustrations are numerous, well selected, and of good quality.

This represents a truly major contribution to the literature on pathological anatomy. Nothing else so comprehensive is available by an American author. A wealth of information has been collected, analyzed, and correlated. The opinions on the whole are well balanced and authoritative. No adverb less than monumental characterizes this work. It is an important and lasting contribution to pathological anatomy, which is highly recommended for students of disease.

Electron Microscopic Atlas of Normal and Leukemic Human Blood. By Frank N. Low, Ph.D., and James A. Freeman, Research Assistant and Public Health Training Fellow. Price, $25. Pp. 347, with illustrations. McGraw-Hill Book Company, Inc., 330 W. 42d St., New York 36, 1958.

This atlas includes two hundred fourteen electron photomicrographs of normal and leukemic human cells in the peripheral circulating blood. The samples were prepared by a rather simple technique, which involves minimal technical manipulation. Apparently, few artifacts are introduced by this method, which largely separates leukocytes and erythrocytes. Enough illustrations of each cell type are included to give an appreciation of the variation in appearance of the different cells. Characteristics of normal leukocytes in the peripheral blood are illustrated with eighty-six micrographs. Circulating leukocytes found in granulocytic, myeloblastic, stem-cell, lymphocytic, and monoblastic leukemia and in multiple myeloma are presented in 123 electron micrographs. Five micrographs illustrate circulating nucleated erythrocytes. The photomicrographs are of excellent quality and are extremely well reproduced. They have been selected from four thousand separate negatives obtained in the study of fifty-one blood samples from thirty-six persons. It is regretable that photomicrographs from a greater number of samples are not included. The published micrographs are from twenty-one blood samples from fifteen persons; forty-two micrographs are included from one blood sample!

The text is primarily descriptive and is generally clear and fairly complete. However, there are a few inconsistencies. It is emphasized in the introduction that "no clear distinc-

tion between eosinophils and basophils (can) be made at the present time." Later, with only slight mention of interpretive difficulties, "basophilic forms" are confidently identified. The use of the same label (MB) for both "myeloblast" and "monoblast" is somewhat confusing. The choice of the term "stem cell" to describe the predominant cell type in a case of treated stem-cell leukemia seems ill advised, since the authors emphasize that this cell "possesses mature characteristic" and does not resemble the typical myeloblasts seen in untreated stem-cell leukemia. The intriguing possibility that the morphology of these "stem cells" has been altered by therapy certainly deserves careful investigation.

These are only minor annoyances which detract but slightly from the value of this atlas. It should be useful to anatomist, pathologist, hematologist, and investigator who are interested in the morphology of circulating human blood in normal and abnormal situations.

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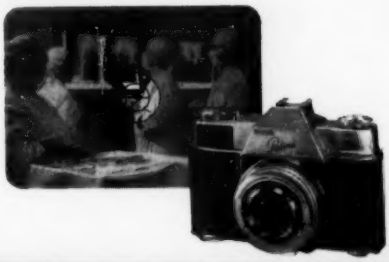

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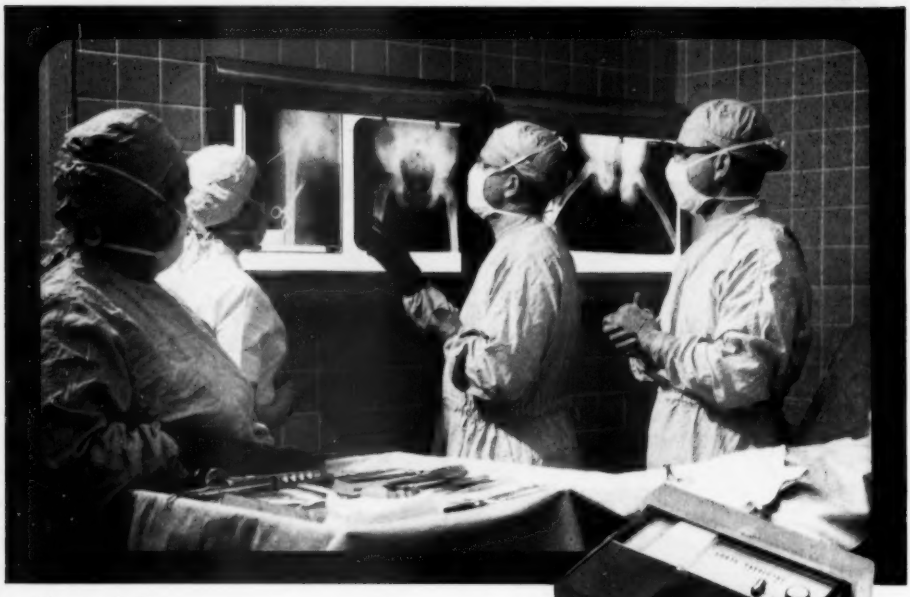

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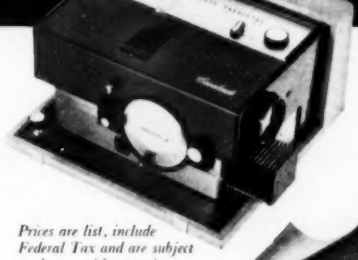

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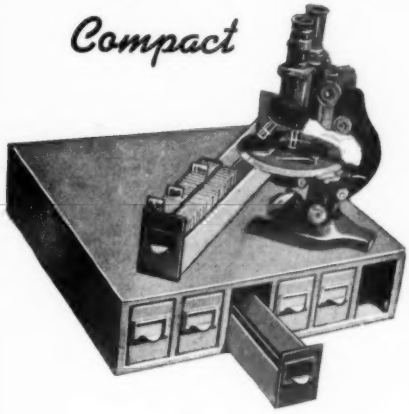

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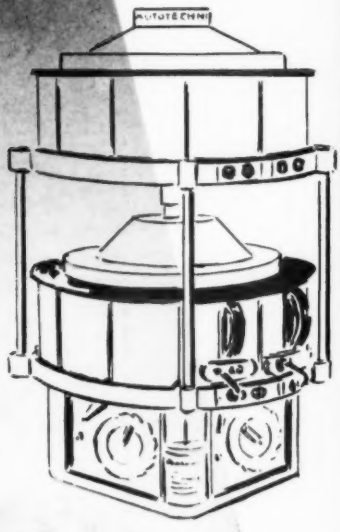

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